THE BIOGEOCHEMICAL CYCLING OF SULPHUR COMPOUNDS

by

Simon Charles Baker, B.Sc. (Reading)

This thesis is presented for the Degree of Doctor of Philosophy, in the Department of Biological Sciences, University of Warwick

February 1992

To my family

DECLARATION

The work contained in this thesis was the result of original research conducted by myself under the supervision of Prof. D. P. Kelly and Dr. J. C. Murrell. All the sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been submitted for any previous degree.

ACKNOWLEDGEMENTS

I would like to thank Don Kelly and Colin Murrell for their help over the last three years, as well as the other members of Biological Sciences at Warwick, including, Ann Wood, Lesley Phillips, Penny Bramwell and all the technicians.

My particular thanks go to Rachel Neal for keeping me sane, Richard Neal for the loan of a computer, without which writing would not have been so easy, and to Alex Baker and Gill Proctor for help with 1-2-3 and accomodation.

Thank you also to Wim Meijer and Lubbert Dijkhuizen of Groningen University, in which part of this thesis was written. The help of postdoctoral workers in several laboratories around the world has been invaluable.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS USED THROUGHOUT THE TEXT

CNN	Cloud condensation nucleii
CO_2	Carbon dioxide
cpm	Counts per minute
CS_2	Carbon disulphide
D_c	Critical dilution rate
DEDS	Diethyl disulphide
DES	Diethyl sulphide
DMA	Dimethylammonium hydrochloride
DMDS	Dimethyl disulphide
DMS	Dimethyl sulphide
DMSP	Dimethyl sulphonium propionate
DNA	Deoxyribonucleic acid
DNP	2,4 dinitrophenol
EDTA	Sodium ethylenediamine tetraacetate
ESA	Ethane sulphonate
FID	Flame ionisation detector
μmax	Maximum growth rate
FPD	Flame photometric detector
GC	Gas chromatograph
GC/MS	Gas chromatograph/mass spectrometer
HPLC	High performance liquid chromatography
HPR	Hydroxy pyruvate reductase
HPS	Hexulose phosphate synthase
LC50	Lethal concentration of inhaled gas causing death in half the affected population
l	Litres

m	Metres
M	Molar
mA	Milliamps
M2 buffer	Carbon source-free MinE
MDH	Methanol dehydrogenase
$MeNO_2$	Nitromethane (CH_3NO_2)
MinE	Minimal medium E
MinE-S	Minimal medium E without sulphate
MMA	Methylammonium hydrochloride
MMS	Monomethyl sulphate
MMSA	Methylmethane sulphonate
MPA	Methane phosphonate
MSA	Methane sulphonic acid
MT	Methanethiol
NA	Nutrient agar
$NAD(P)^+$	nicotinamide dinucleotide phosphate or nicotinamide dinucleotide
NAD(P)H	Reduced nicotinamide dinucleotide phosphate or reduced nicotinamide dinucleotide
NAD^+	Nicotinamide dinucleotide
NADH	Reduced nicotinamide dinucleotide
$NADP^+$	nicotinamide dinucleotide phosphate
NADPH	Reduced nicotinamide dinucleotide phosphate
NH_2MSA	Aminomethane sulphonate
OD_x	Optical density of a solution measured at x nm, where $x < 1000$ nm.
RNA	Ribonucleic acid

RNase	Ribonuclease
rpm	Revolutions per minute
RUBISCO	Ribulose-1,5-bisphosphate carboxylase (carboxy dismutase)
RuBP	Ribulose bisphosphate
RuMP	Ribulose monophosphate
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Saline sodium citrate solution
TE buffer	buffer
TMA	Trimethylammonium hydrochloride
TMS	Trimethylsulphonium chloride
TMSI	Trimethylsilylimidazole
TvAM	Acidic *Thiobacillus versutus* medium
TvM	*Thiobacillus versutus* medium
w/v	Concentration expressed as weight of substance in grammes per 100 millilitres solvent
v/v	Concentration expressed as volume of solute in millilitres per 100 millilitres of solvent

In addition, chemical formulas for inorganic and organic molecules are used throughout the text.

SUMMARY

Organosulphur compounds play an important role in the cycling of sulphur, and may even have influence in meteorological changes. Biogeochemical transformations involving carbon disulphide, diethyl sulphide and methane sulphonate (MSA) are discussed with particular reference to the role of bacteria. The aim of the work presented is to elucidate the mechanisms by which bacteria metabolize these sulphur compounds.

Isolates from a collection assembled by C. Harfoot were heterotrophic in nature, but had the ability to grow with diethyl sulphide and carbon disulphide as sole sources of carbon and energy. An attempt was made to identify the Gram positive bacteria using numerical taxonomy, but the results were negated by the discovery that the cultures were mixed. It is suggested that the growth by heterotrophic bacteria in media containing diethyl sulphide was supported by the primary action of microaerophillic thiobacilli.

Isolation procedures are described for bacteria capable of growth on carbon disulphide and methane sulphonate. The former was not a success, due to a combination of the toxicity of the substrate and the unusual culture techniques required, but impure cultures catalysed the disappearance of carbon disulphide at rates exceeding those previously recorded.

Nine isolates were easily obtained using MSA as sole source of carbon, energy and sulphur, with a variety of morphological features. 23 type cultures were also tested for growth on MSA. None of them grew or could oxidise MSA. One new isolate (M2), a Gram negative rod, was selected for further study. The limited taxonomic data available for the organism did not lead to any definitive identification of the strain, but it is postulated that the strain represents a new genus of bacteria.

M2 grew methylotrophically on MSA with the production of acid, in the form of sulphurous acid. This was non-biologically oxidised to sulphuric acid. The organism could grow on C ₁compounds, as well some organic acids and sugars. Carbon from C ₁ metabolism was assimilated via the serine pathway. No conclusive evidence could be found for the existence of ribulose bisphosphatecarboxylase activity. Isolate M2 possessed methanol dehydrogenase and formate dehydrogenase when grown on C ₁ compounds (including MSA), but not when grown on pyruvate.

Two alternative primary cleavages of MSA are suggested, resulting in methanol or formaldehyde as the primary intermediate, but no evidence could be found to support the sole existence of either. The approach to differentiate between the pathways focused on the inhibition of methanol oxidation in whole cells. MSA oxidation could not be detected in cell-free extracts.

The ease with which M2 was isolated suggests ubiquity of MSA users in the environment, a postulate reinforced by the isolation of similar cultures from a variety of habitats by other workers. The terrestrial fate of MSA, which is found in rainfall, appears to be oxidation to carbon dioxide and sulphate by methylotrophic soil bacteria.

THE BIOGEOCHEMICAL CYCLING OF ORGANOSULPHUR COMPOUNDS

Yet one caution let me give by the way to my present or future reader - that he read not the symptoms or prognostics of the following tract, lest, by applying that which he reads to himself, aggravating, appropriating things generally spoken, to his own person (as melancholy men for the most part do), he trouble or hurt himself, and get, in conclusion, more harm than good. I advise them therefore warily to peruse that tract.

Robert Burton, (1621), Introduction. In *The Anatomy of Melancholy*, Oxford. Source: Umberto Eco (1989), *Foucault's Pendulum*, Secker and Warburg, London.

CHAPTER 1:
INTRODUCTION

1.1 The biogeochemical sulphur cycle

The concept of a particular atom, compound or group of compounds being transformed by geochemical, anthropogenic and biological processes on a global scale is not new: the cycling of water and carbon and nitrogen have been recognised in biology and geography. A similar cycle can be constructed for sulphur, which at the most basic level is much like that of carbon (figure 1.1.1). The primary difference is that unlike carbon dioxide (CO_2) and photosynthesis, biological agents do not play as great a

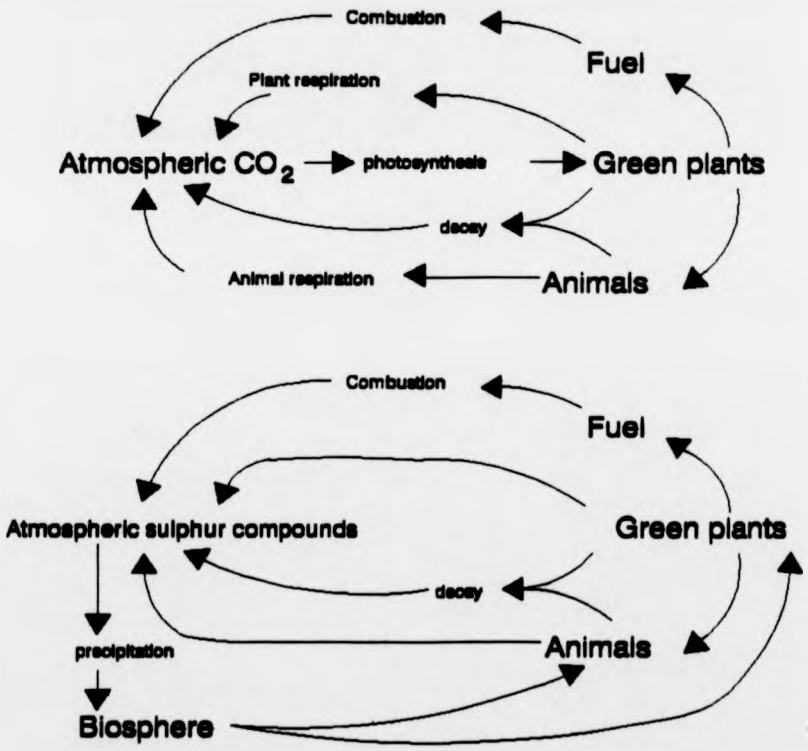


Figure 1.1.1 A comparison of the cycling of carbon and sulphur.

part in the removal of sulphur compounds from the atmosphere to a terrestrial form.

Once specific geochemical, biological or anthropogenic reactions are considered, both carbon and sulphur cycles become far more complicated, but sulphur poses particular problems with its multiple oxidation states, giving rise to a plethora of compounds that can be deemed to be important in the atom's cycling. Without question, the most significant volatile carbon compound in the atmosphere is CO_2, serving as a link between terrestrial and marine environments, but there is no direct sulphur equivalent. In prioritising volatile atmospheric sulphur compounds, sulphur dioxide (SO_2), hydrogen sulphide (H_2S), carbonyl sulphide (COS), carbon disulphide (CS_2), dimethyl sulphide ($CH_3.SCH_3$; DMS) dimethyl disulphide ($CH_3.SSCH_3$; DMDS), methane thiol ($CH_3.SH$; MT) and many others may be considered, dependent on the geographical location under examination. The nature of sulphur's chemistry may mean that non-volatile molecules, such as methane sulphonate and sulphate may also be present in quantity in the atmosphere as dissolved aqueous ions.

The origins of these volatile sulphur compounds are varied. "Natural" sources include the planetry chemical reactions that arise from volcanoes, fumaroles and other geothermal vents, emissions as a result of forest fires and the sulphur chemicals released biologically from both land masses and the oceans. Anthropogenic sources include the combustion of fossil fuels, particularly coal, and the use of sulphur compounds during the manufacture of chemical substances. The

estimated emission rates from natural sources of the most abundant atmospheric sulphur compounds are shown in table 1.1. The figures taken from the review by Kelly and Smith (1990) will probably be subject to a reduction as the diversity and numbers of samples increases.

	Sulphur compound released (Tg S $year^{-1}$)						
Source	SO_2	H_2S	DMS	DMDS and others	CS_2	COS	Total
Oceanic		0-15	38-40	0-1	0.3	0.4	38.7-56.7
Salt marsh		0.8-0.9	0.58	0.13	0.07	0.12	1.7-1.8
Inland swamps		11.7	0.84	0.2	2.8	1.85	17.4
Soil and plants		3-41	0.2-4.	1	0.6-1.5	0.2-1.0	5.0-48.5
Burning of biomass	7	0-1		0-1		0.11	7.1-9.1
Volcanoes and fumaroles	8	1		0-0.02	0.01	0.01	9.0
Total	15	16.5-70	39.6-45.4	1.3-3.4	3.8-4.7	2.7-3.5	78.9-142.6

Table 1.1 Ranges of estimated rates of emission of volatile sulphur compounds to the atmosphere from natural sources (from Kelly and Smith, 1990).

The rates of emissions of sulphur chemicals from anthropogenic sources are difficult to extrapolate to a global, yearly level, as the atmospheric gaseous composition above populated areas tends to vary enormously with time and position. For example the atmosphere above viscose rayon factories may be subjected to the emission of 100-300 kg CS_2 per tonne of silk produced (Hoeven *et al*, 1986; Katalyse, 1987), which could lead to a gross over-estimation of anthropogenic CS_2 if samples taken anywhere near the factory are used for a global nterpretation (For this reason, samples used to assess natural levels of sulphur compounds are normally taken in remote areas.). However, it is

believed that the emission of sulphur compounds (including sulphur dioxide) from anthropogenic sources is at approximately the same rate as that from natural sources, up to 142 Tg S $year^{-1}$.

Although a great variation can be anticipated according to the immediate locality and some care must be taken in interpretation due to the innacuracies of the measurements involved, some estimation was made of the concentrations of volatile sulphur compounds in air and water (Table 1.1.2), again by Kelly and Smith (1990).

Compound	Freshwater ($\mu g\ l^{-1}$)	Seawater ($ng\ l^{-1}$)	Salt Marsh ($ng\ l^{-1}$)	Air ($pg\ l^{-1}$)
DMS	62-70	3-310	60-3800	6-200
DMDS	*	14-19	94-282	*
MT	*	14-19	48-144	*
CS_2	*	0.5-19	76-228	20-1200
COS	*	14-19	60-180	510

Table 1.1.2 Ranges of concentrations of some volatile organic sulphur compounds in the environment. * = no data available, but undoubtedly present in the environment, albeit at low concentration. Data from Kelly and Smith, (1990).

The main change in the concept of sulphur cycling has arisen from the realisation that organic sulphur compounds play at least as important a part as inorganic molecules such as hydrogen sulphide and sulphate. The microbiology and ecology of these compounds has recently been reviewed by Kelly and Smith (1990). In this review they noted several compounds were worthy of further study, particularly methane sulphonic acid and carbon disulphide. The former was also cited by a

workshop on biogeochemical cycling as deserving more attention (Galloway *et al.*, 1985).

1.2 Carbon disulphide

1.2.1 Introduction

Carbon disulphide (CS_2) is a significant volatile sulphur compound in the atmosphere and pedosphere, both as a "natural" product and a pollutant (figure 1.2.1). The molecule is composed of a central carbon atom associated by double bonds to two sulphur atoms. At room temperature it is a volatile liquid (boiling point of 46.2 °C, density 1.26, flash point -30 °C [Courtaulds plc 1987]). When pure this toxic compound is said to have an ethereal odour, but more commonly trace organosulphur contaminants give the liquid a strong, unpleasant smell.

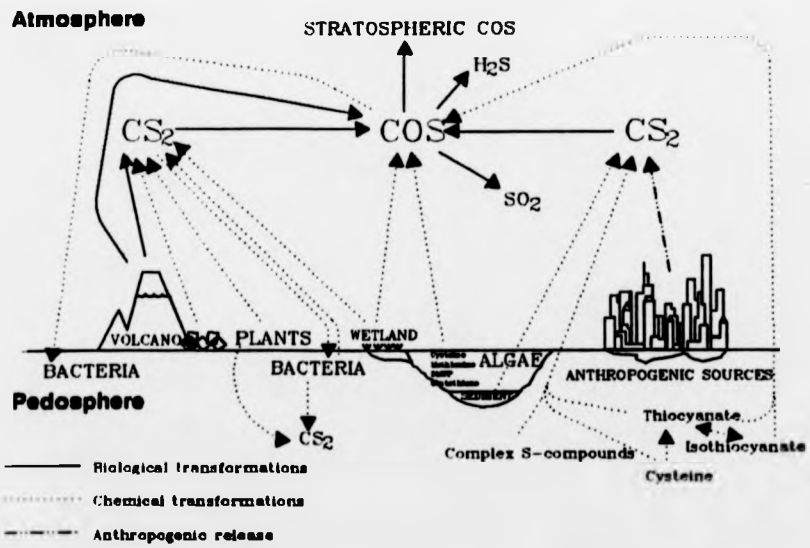


Figure 1.2.1 Biogeochemical transformations of carbon disulphide.

Industrially, CS_2 has been used in its capacity as an organic solvent and as a reactant for many chemical processes. The main industrial user is the cellulose industry for the manufacture of viscose silk (also known as artificial silk or Rayon). The other major uses of CS_2 are listed in table 1.2.1. An unusual use of CS_2 was as a dog repellent, marketed in the USA - a can of CS_2 could be thrown in the face of the offending dog, though which specific quality of CS_2 succeeded in driving the animal away and prevented it coming back (perhaps terminally) is

Production process	Tonnes p.a.
Manufacture of viscose	86 300
Manufacture of cellophane	23 000
Manufacture of carbon tetrachloride	6 200
Manufacture of tetrahydro-2H-1,3,5-thiadiazine-2-thione (biocide)	68 000
Manufacture of tetramethyl-thuradisulphide (vulcanisation accelerator) Manufacture of 2-mercpto-2-imidazoline (vulcanisation accelerator) Manufacture of dithiocarbamates (rubber and polymer protection)	22 500
Xanthate flotation auxiliary	5 400
Polymerisation inhibitor in vinyl chloride manufacture	--
Polymerisation inhibitor in neoprene plastic manufacture	--
Sulphide agents for the exhibition of rare earths	--
Solvent and artificial fabric spinning solutions	--

Table 1.2.1 Industrial applications of CS_2 and consumption in Western Europe
Consumption figures taken from Anon (1986)

debatable. Detailed surveys of all the possible uses and syntheses involving CS_2 are given by Timmerman (1978) and Thömel (1987).

1.2.2 Effect of carbon disulphide on the biosphere

CS_2 has been demonstrated to be toxic to every organism against which it has been tested, often at very low concentrations. These include representatives from most of the major taxa (See table 1.2.2).

CS_2 has many toxic effects to humans, including heart disease, sterility, psychological changes and even death (Anon 1986). Long term exposure safety limits are as low as 10 ppm (Anon 1986). CS_2 could enter the human food chain in several ways, apart from

Species	Organism Type	LC_{50} 48hr	50% growth inhibition
Photobacterium phosphoreum	Bacterium	--	340
Chlorella pyrenoisoda	Algae	--	21
Daphina magna		2.1	--
Gambusia affinis	Fish	135	--
Homo sapiens	Mammal	0.03*	

Table 1.2.2 Effect of carbon disulphide on various organisms. All figures are expressed as mg l^{-1}. (From Courtaulds plc, 1987)

* Recommended minimum exposure limit

absorption into organism from the atmosphere. In America it is a common practice to fumigate grain with a mixture of CS_2 and carbon tetrachloride (Peters *et al.* 1986). CS_2 is also used in fish meal processing (Courtaulds plc 1987). Since CS_2 has a relatively high solubility in water for an organic solvent (2.1 g l^{-1}, Lay *et al.* 1986), if the grain or meal is slightly damp, some CS_2 will remain in the foodstuff

after the bulk has been removed (Daft 1987).

Despite its toxicity, CS_2 is an important intermediate in the biogeochemical cycling of organosulphur compounds and present throughout the environment, albeit at low concentrations (see table 1.1.2).

1.2.3 Transformations in the biogeochemical cycling of carbon disulphide

1.2.3.1 Anthropogenic sources of carbon disulphide

Between 1 and 1.7 million tonnes of CS_2 were manufactured annually between the years 1977 and 1987 (Timmerman 1978; Thömel 1987). The extent of anthropogenic release can be seen from comparing atmospheric concentrations in remote and populated areas; CS_2 concentration above industrial districts is at least twice as high (see table 1.2.2). Of particular note is the viscose industry, consuming 65% (globally, [Courtaulds plc 1987]) of manufactured CS_2 and releasing 95% of this amount as a waste product during the viscose manufacturing process (Hoeven *et al.* 1986; Katalyse, 1987). The need to ventilate industrial premises sufficiently to keep CS_2 concentrations down to safe levels means that all manufacturing processes involving the use of CS_2, including its own manufacture, release the sulphide to the atmosphere to a greater or lesser extent. Other activities such as oil refining and using coal blast furnaces result in the release of CS_2 (Ackerman *et al.* 1980). In their review of the CS_2 minicycle, Kelly and Smith (1990) suggested that an overestimate of the anthropogenic

contribution could be 1 million tonnes annually. This figure could be reduced by at least a half as it takes no account of scrubbing of exhaust air.

1.2.3.2 Biological sources of carbon disulphide

Few studies have been made on the biological emission of CS_2 and so a global figure is hard to estimate. Evidence of biogenic CS_2 has been found in anaerobic marine sediments (Lovelock, 1974; Wakeham *et al.* 1984), Antarctic lake waters (Deprez *et al.* 1986) and in terrestrial sulphur emissions. (Guenther *et al.* 1989). The production of CS_2 has also been noted from soils supplemented with cysteine, homocysteine, lanthionine, plant materials, manures and sludges (Banwart and Bremner, 1976; Bremner and Steele, 1978). Marsh lands have so far been attributed with the greatest production rates (see table 1.2.1). The marsh plant *Spartina alterniflora* is said to be the main origin (Aneja *et al.* 1979).

Many other higher plants release or contain CS_2. The most extreme case is that of the tree *Stryphnodendron excelsum*, whose location in the rain forest can be pin-pointed by smell alone (Haines *et al.*, 1989). Disturbing the roots and earth around the tree could lead to the release of up to 6 $\mu g\ CS_2\ m^{-2}\ minute^{-1}$. Other plant species with the capacity to emit CS_2 include *Brassica oleracea* (Haines *et al.*, 1989), *Medicago sativum*, *Zea mays*, *Quercus lobata* (Westberg and Lamb, 1984) and *Acacia pulchella* (Whitfield *et al.* 1981). The sulphide's function has been suggested as a biocide to

protect against nematodes and other pathogens, or as a means of regulating rhizosphere sulphate concentration (Haines *et al.*, 1989)

1.2.3.3 Other sources of carbon disulphide

There are many sources of CS_2 with low emission rates. Among the higher ones are the burning of biomass (Eg. forest fires), emission from soils (presumably biological in origin) and from volcanoes. The output of CS_2 to the atmosphere from volcanoes is estimated at 0.01 Tg sulphur per annum, and may be as high as 19 ng l^{-1} in the plume (Rasmussen *et al.*, 1982b; Stoiber *et al.*, 1971). A minor contribution to the atmosphere is from some sulphur-rich rocks.

1.2.3.4 The fate of atmospheric carbon disulphide

Once in the atmosphere CS_2 may be photooxidised to carbonyl sulphide (COS) with a half life of 12 days. COS will eventually contribute to atmospheric sulphur dioxide and hydrogen sulphide levels. (see figure 1.2.1). Alternatively, a minor photooxidation of CS_2 is directly to sulphur dioxide.

1.2.3.5 Biological sinks for carbon disulphide

Little has appeared in the literature on the subject of biological metabolism of CS_2, work mainly being restricted to the compounds inhibitory and toxic properties. Since CS_2 is present in the environment in detectable quantities, it is reasonable to assume that organisms have developed

mechanisms to grow on or detoxify this sulphide. Methanogens have been isolated capable of using CS_2 as a sulphur source (Rajagopal and Daniels, 1986) and a culture isolated by Dr. C.G. Harfoot (C.G. Harfoot and D.P. Kelly, unpublished data) could oxidise the compound to COS (See section 1.3.2). Neither phenomenon was examined in detail.

1.2.4 Mechanisms of metabolism of carbon disulphide

To date two mechanisms of CS_2 metabolism have been described, one that allows rats to metabolize low concentrations of CS_2, minimising the compound's hepatotoxicity, the other providing energy for the growth of a bacterium.

Chengelis and Neal (1987) demonstrated that rat hepatocytes were capable of metabolizing up to 0.2 mM CS_2 and microsomes up to 0.6 mM. Mineralisation of the hydrogen sulphide ion formed proceeds via thiosulphate to sulphate.

A pathway for the microbial metabolism of CS_2 was proposed by Smith and Kelly (1988c). Their data was for the organism *Thiobacillus thioparus* strain TK-m (see fig 1.2.4.2). The route for the mineralisation proposed, initially via a hydrolysis of CS_2 to COS was in contrast to the mammalian pathway deduced by Chengelis and Neal (1987, see above). The hydrolysis by TK-m provided energy for growth, from the oxidation of the sulphide intermediate to sulphate. Although this energy was sufficient to support chemolithoautotrophic growth, yields were very low. In both cases an oxidation was initiated by an NADPH/cytochrome P_{450}-dependent enzyme, to

yield an unstable intermediate (see figure 1.2.4.1). This breaks down in the presence of water to monothiocarbonate, which forms COS with carbonic anhydrase as the catalyst.

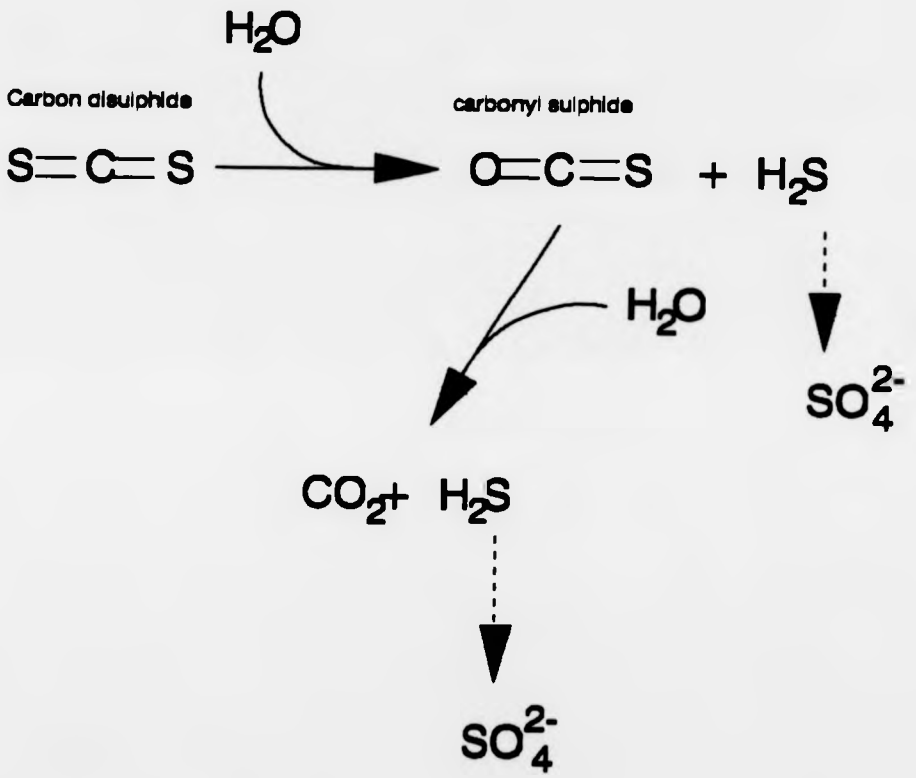


Figure 1.2.4.2 The metabolism of carbon disulphide by Thiobacillus thioparus

1.3 Alkyl sulphides and disulphides

1.3.1 Microbial metabolism of alkyl sulphides

1.3.1.1 DMS and DMDS

The organisms that have been studied in detail that grow with DMS as sole carbon and energy source can be divided into two groups: the thiobacilli and the hyphomicrobia.

1.3.1.1.1 *Thiobacillus* species

Several species of *Thiobacillus* have been isolated capable of using DMS, DMDS, and MT as energy sources for growth (Kanagawa and Kelly, 1986; Smith and Kelly, 1988a, b). These autotrophic organisms completely mineralise DMS and DMDS (Figure 1.3.1.1.1) to CO_2 and sulphate, deriving energy from

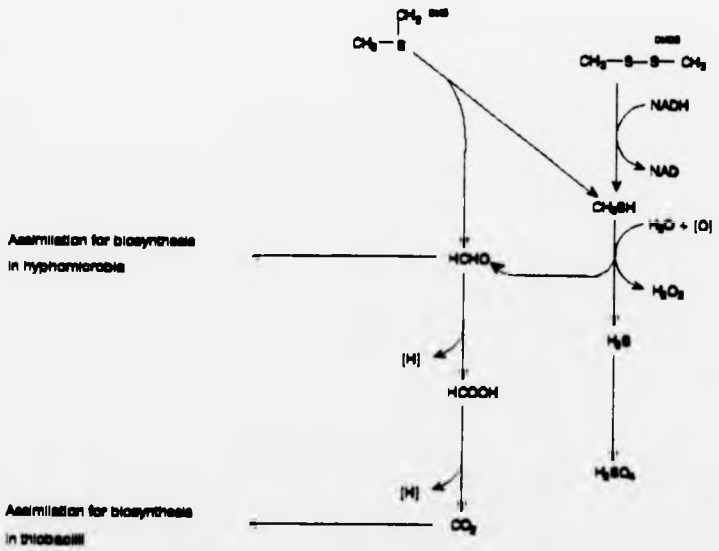


Figure 1.3.1.1.1 The metabolism of DMS by Hyphomicrobium and Thiobacillus species.

the oxidation of the sulphide and the methyl groups. Carbon is assimilated via CO_2 and through the Calvin cycle (see section 7.2.1.2).

However, the first pure culture that was reported to be capable of oxidising DMS assimilated carbon via the RuBP pathway simultaneously operating with a variant of the serine pathway (section 7.2.1.1). The presence of the serine pathway enzymes, however, did not allow methylotrophic growth on C_1 compounds such as MMA, methanol and formaldehyde (Sivelä and Sundman, 1975; Sivelä, 1980).

1.3.1.1.2 *Hyphomicrobium* species

Although the best studied hyphomicrobia were isolated on DMSO (de Bont *et al.*, 1981; Suylen, 1988), they were capable of using DMS, DMDS and MT (Figure 1.3.1.1.1), bya similar metabolic route to *Thiobacillus thioparus* strains E6 and TK-m (Kanagawa and Kelly, 1986; Smith and Kelly, 1988a, b; Kelly, 1988). However, the hyphomicrobia are capable of using the methylated sulphides as sole carbon *and* energy source, assimilating carbon as formaldehyde via the serine pathway. They are incapable of autotrophic growth and do not possess a Calvin cycle. Although the mechanism of the oxidation of sulphide is unknown in the hyphomicrobia, these organisms can obtain energy from the oxidation.

1.3.1.1.3 Other organisms

Recently, Visscher and van Gemerden (1991) examined a purple sulphur bacterium (*Thiocapsa roseopersicina*) and described

it as capable of growth on DMS. These slightly misleading description refers to the organism's ability to use DMS as an electron donor for CO_2 fixation during otherwise conventional autotrophic growth. The organism oxidised DMS to DMSO stoichiometrically, and this may have some relevance in marine environments rich in DMS. Similar oxidations have been described in impure cultures of phototrophic purple sulphur bacteria by Zeyer *et al* (1987).

1.3.1.2 Diethyl sulphide (DES) and diethyl disulphide (DEDS)

The higher alkyl sulphides have not been recorded as present in the atmosphere, but are conceivably present at low concentrations. They are normally associated withodours such as garlic (Sparins *et al*, 1988), which contains dipropyl sulphide, and onions (Kjaer, 1977), which contain propylmethyl disulphide.

The chief interest in the microbiology of DES and DEDS lies in relation to the comparative metabolism of these compounds with regard to DMS and DMDS. Gram negative bacteria, isolated by C. Harfoot (Kelly and Smith, 1990), have been isolated from diverse southern hemisphere locations, and are capable of growth on DMS, DMDS and DES. These cultures also oxidised longer chain alkyl sulphides. Further work on these isolates is described in chapters 2,3 and 5.

1.3.2 The role of DMS in terrestrial/marine sulphur exchange

When considering a system of such magnitude and complexity

as the sulphur cycle, it is not surprising that the apparent importance of various compounds changes according to the latest research. The changeable nature of the ideas of sulphur cycling are compounded by the necessity of examining relatively small parts of the cycle, influenced by many factors, and trying to extrapolate to global proportions. Both these problems led to the adoption of H_2S as the most important gaseous sulphur compound, linking terrestrial and marine environments (Rodhe and Isaksen, 1980). This was mainly because of its ease of detection and abundance in certain areas such as swamps and salt marshes. H_2S appears herebecause of the reduction of sulphate. Data from these environments led to an estimate of annual global production of H_2S of 142 Tg (Robinson and Robbins, 1970). This maximised global estimate was questioned by Rasmussen (1974), who postulated that although the cycling of sulphate sulphur as H_2S is important in anaerobic environments, and at the interface of these environments with soil and water, H_2S is primarily a factor in closed cycles. On this basis, it only contributes 5-58 Tg S $year^{-1}$ to the overall atmospheric cycle. The turnover of sulphur through H_2S is still thought to exceed the release, especially if the gas has to pass through biologically active strata above its anaerobic production site.

Improved chromatographic methods indicated alternative candidtaes for the most important gaseous atmospheric sulphur compounds, and confirmed that H_2S was not as abundant as was first thought. Attempts to detect

concentrations of H_2S consistent with the model failed. Instead, gases such as CS_2, COS and methylated sulphides were present. The sources of these compounds were at first not clear, although Challenger (1951) reported that many living systems produced DMS, including marine algae. This led Lovelock *et al.* (1972) to propose that DMS had the role previously given to H_2S.

1.3.3 The origins of DMS

1.3.3.1 Anthropogenic sources

Although biogenic and anthropogenic input of organosulphur gases into the atmosphere are thought to be approximately equal, it is difficult to identify specific industries or activities that result in the release of DMS into the atmosphere. The wood pulping industry, oil refineries (Kanagawa and Mikami, 1989) and mushroom cultivation (Derikx *et al*, 1990) have been mentioned in connection with DMS, but no quantitative data exists.

1.3.3.2 Terrestrial biogenic sources

The total production of DMS from terrestrial sources is estimated at 4.5 Tg S $year^{-1}$. DMS is produced by *Spartina* and probably by microbial action in salt marsh and marine sediments (Kelly and Smith, 1990; Taylor and Kiene, 1989).

Kiene and colleagues have extensively described the behaviour and turnover of DMS in salt marsh sediments and other coastal environments (Kiene and Visscher, 1987; Taylor and Kiene, 1989; Kiene and Bates, 1990). Some DMSP,

presumably of vegetable origin, is converted to DMS anaerobically and DMSO reduced biologically also to produce DMS. MT was also found to undergo methylation to form DMS. Thus in anaerobic muds, a complex interaction between methylated sulphides arises, presumably due to the presence of bacteria (Figure 1.3.3.2).

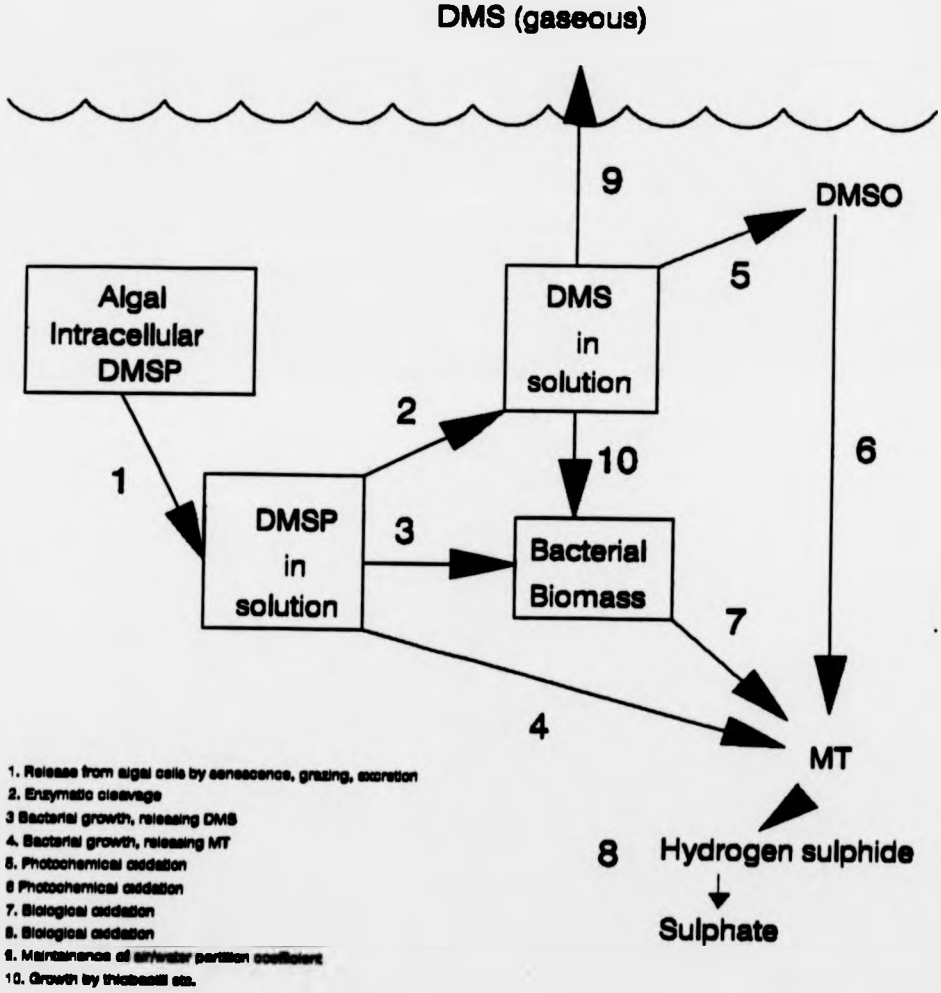


Figure 1.3.3.2 Transformations through DMS in seawater and marine sediments.

DMS can act as a substrate for methanogens and sulphate reducing bacteria in salt marsh sediments (Kiene, 1988; Kiene and Capone, 1988) and up to 28% of methane released from one site was recorded as having its origins in DMS (Kiene, 1988). DMS is also released when DMSO is used as an anaerobic electron acceptor by various bacteria (Zinder and Brock, 1978; King *et al*, 1987).

1.3.3.3 Marine biogenic sources

The correlation of DMS in the atmosphere with marine phytoplankton was pointed out by a number of authors (Lovelock *et al*, 1972; Rasmussen, 1974; Nguyen *et al*, 1978; Barnard *et al*, 1982) and this was linked to the enzymatic cleavage of DMSP (yielding DMS and acrylate), first demonstrated in marine macroalgae by Challenger (1951). The function of DMSP in algae has not been absolutely determined, but may have an osmoregulatory function analogous to that of quaternary nitrogen compounds in higher plants and marine invertebrate animals (Dickson *et al*, 1980). There is evidence that DMSP may act as a methyl donor (Maw and du Vigneaud, 1948; Dubnott and Borsook, 1948). The product of DMSP cleavage, acrylate, has been assigned an antibiotic role (Sieburth, 1960).

DMSP is the major sulphur compound in most algae (Suylen, 1988; Kiene and Taylor, 1988a, b). Whatever the role of DMSP, it is certainly the major source of marine DMS. The proposed mechanisms of DMS release from algae have included senescence (Zinder *et al*, 1977; Bechard and Rayburn, 1979),

a continuous slow cleavage by algal enzymes (Bechard and Rayburn, 1979), grazing by zooplankton (Dacey and Wakeham, 1986) and saline or nitrogen stress (Vairavamurthy *et al*, 1985; Turner and Liss, 1987).

A definite correlation between concentrations of DMSP,DMS and phytoplankton in the sea has been established (Cline and Bates, 1983; Barnard *et al*, 1984; Wakeham *et al*, 1984; Ayers and Gras, 1990), and work by Kiene (Kiene, 1990; Kiene and Bates, 1990) has shown that the turnover of DMS in marine environments far exceeds its release. Form this, and work by Dacey and Wakeham (1986), it can be seen that the production of DMS and DMSP is a function of marine bacteria and algae. Taking the DMS consumption rates given by Kiene and Bates (1990), it can be calculated that the oceans have the potential to consume approximately 695 Tg S $year^{-1}$ while around 38-40 Tg S $year^{-1}$ are released into the atmosphere from the global marine environment.

The oceans are not the sole source of DMS: it is detectable in most environments (see figure 1.3.3.3). Emissions have been detected from soils (Hitchcock, 1975) and forests (Hitchcock, 1975; Lovelock *et al*, 1972) as well as mudflats (Aneja *et al* 1979a) and salt marshes (Steudler and Peterson, 1984; Aneja *et al* 1979a).

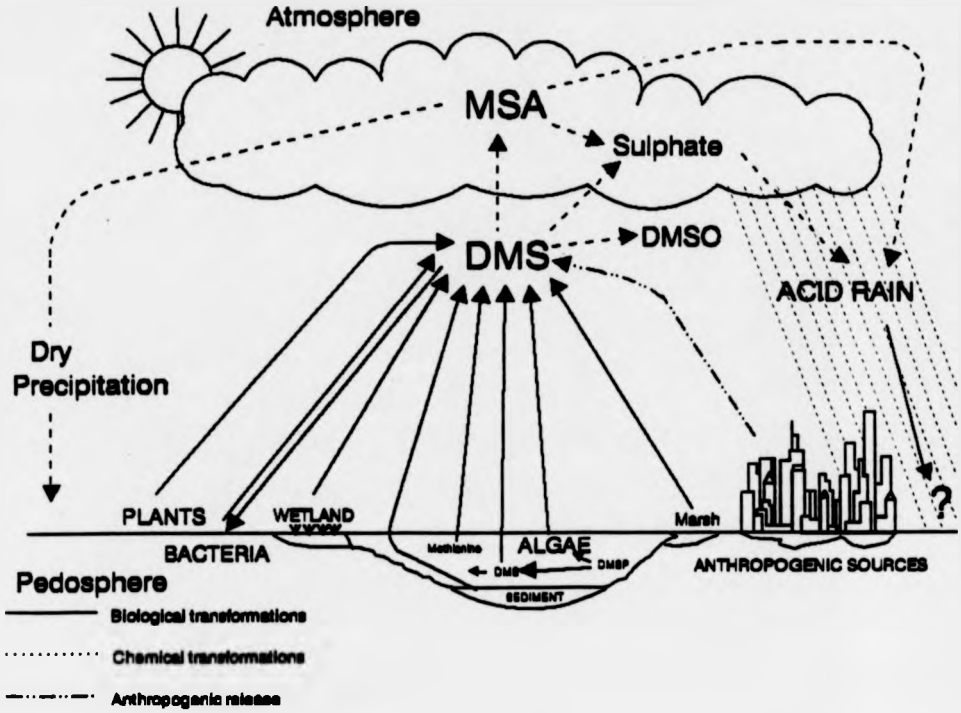


Figure 1.3.3.3 Biogeochemical transformations in the cycling of sulphur through DMS.

1.3.3.4 Non-biological sinks for marine DMS

DMS may be oxidised photochemically in marine environments, or adsorb to particles and fall to the sea bed (Kiene and Bates, 1991). However, the collective rate of these processes is far exceeded by turnover to the atmosphere and by the biological processes outlined by Kiene and Bates (1991).

1.3.4 The atmospheric oxidation of DMS

DMS is rapidly oxidised in the atmosphere by hydroxyl radicals to a variety of products including MSA, sulphate and DMSO (Andreae *et al*, 1988; Cocks and Kallend, 1988). The photochemistry of the reaction (summarised in figure 1.3.4)

is still not completely understood, but up to 50% of DMS may be converted to MSA (Kelly and Smith, 1990) with sulphate another major product. These molecules are solids, suspended as particles in the atmosphere. As such they act as points around which water can condense, with dust *etc*. These cloud condensation nuclei (CNN) play an important role in the formation of clouds and thus the earths albedeo (Charlson *et al*, 1987; Bates *et al*, 1987a). The ultimate influence of the algal/DMSP/DMS/MSA/cloud transformations on the light intensity reaching the algae could suggest some sort of global feedback (Gibson *et al*, 1988; Charlson *et al*, 1987), but the contribution of atmospheric oxidation products of DMS to the numbers of CNN, is difficult to assess in comparison to the other sources of suspended solids (eg. sulphate from SO_2, sea-spray sulphate, air-borne dust etc).

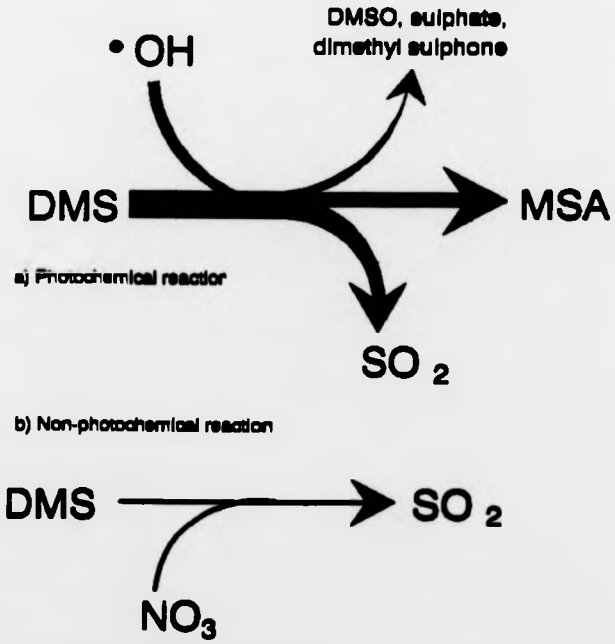


Figure 1.3.4 Photooxidation products of atmospheric DMS

The products of DMS photooxidation have also been implicated in acidity of meteoric precipitation (Nriagu *et al*, 1987; Pearce, 1988; Cocks and Kallend, 1988), but again this is difficult to assess with any confidence with regard to other *natural* sources of sulphuric acid, let alone anthropogenic SO_2. The amounts of MSA measured in Antarctic ice (Saigne and Legrand, 1987; Saigne *et al*, 1987) without variation over the past 10,000 years seemed to suggest that recent increases in amounts of acid rainfall originate from sources other than algal DMS. In the Eastern Pacific Ocean, turnover times for DMS due to biological removal were generally over ten times faster than turnover to the atmosphere (Kiene and Bates, 1991). Therefore, the "health" of the ocean is probably significant in keeping DMS emission and thus acid rainfall to a minimum.

1.4 Methane sulphonic acid

1.4.1 Methane sulphonic acid as a key compound in sulphur cycling

There seems to be a lack of data and some controversy over the fate of MSA in the atmosphere. It is frequently mentioned as an oxidation product of DMS, but little attempt has been made to integrate the compound into the global sulphur cycle. That some MSA is deposited onto the earth is beyond doubt, but even in a publication by leading atmospheric chemists (Galloway, 1985), MSA is shown to be both entirely deposited terrestrially, or a

certain percentage converted to unknown compounds before deposition as sulphate. The precipitation of MSA allows the sulphur released as DMS to enter the pedosphere again, and continue the cycle. To what form the MSA is converted in the next phase of the cycle has, up until now, been unknown.

MSA is a strong, stable acid, so it must be reasonable to assume that a large percentage reaches the earth as acid or combined with compounds present in rainfall as a sulphonate. Work by Saigne and co-workers (Saigne and Legrand, 1987; Saigne *et al*, 1987) led to the conclusion that deposition of MSA has been happening for at least 10,000 years. Their data were gained by measuring MSA concentrations at different depths of Antarctic ice. However, in more biologically active environments, the input of such large amounts of carbon and sulphur must have an effect on the microflora.

1.4.2 Occurrence and biological interaction

The input of MSA into marine and terrestrial environments must have some effect. This has been recognised by many workers (Kelly and Smith, 1990; Chatfield *et al*, 1985), but the literature available on the biological transformations of MSA is limited. *Chlorella fusca* (Biedlingmaier and Schmidt, 1986) can use n-alkane-1-sulphonic acids (C_1-C_8) as sulphur sources for growth, using ethane sulphonic acid (ESA) preferentially. However MSA appeared to inhibit the uptake of sulphonic acids and

was the least well-used sulphur source of those tested. MSA has also been identified as a sulphur source for some bacteria (Cook and Hutter, 1982). The scant information on MSA is surprising in view of the large amounts of MSA that are supplied to bio-active environments, estimated to be as much as 52 Tg $year^{-1}$ (Kelly and Smith, 1990; Baker *et al*, 1991).

1.4.3 The role of bacteria in MSA cycling.

Although many sulphonates are considered xenobiotics (Krauss and Schmidt, 1987) and ethyl- and methyl-esterified MSA are used as powerful mutagens, the chemical properties of MSA itself would seem to make it an ideal substrate for bacteria. The carbon-sulphur bond could be split and the sulphur oxidised to yield energy during autotrophic growth or the methyl group oxidised by a methylotroph. Organisms using MSA should be present in a variety of habitats since rain or dry precipitation falls on the majority of marine and terrestrial locations. The concluding chapters of this thesis present data showing the isolation of a bacterium capable of using MSA as a sole source of carbon and energy, and some information on the mechanisms by which it achieves this.

Without data referring to the fate of MSA in the terrestrial environment, the sulphur cycle remains incomplete. For this reason, the paper describing the isolation of the first bacterium using MSA as sole source of carbon and energy (the isolation is described in more

detail in this thesis) was entitled "Microbial degradation of MSA: a missing link in the biogeochemical sulphur cycle" (Baker *et al.*, 1991).

1.5 The objectives of the work presented

1.5.1 The microbiology of carbon disulphide

CS_2 supports only poor growth of *Thiobacillus* TK-m. It was hoped that enrichment and isolation procedures would yield bacterial strains, using CS_2 as sole energy and/or carbon source, more amenable to biochemical procedures. The pathways by which this process occurred were to be determined.

1.5.2 The Harfoot culture collection

A variety of apparently heterotrophic bacteria isolated in New Zealand and Antarctica appeared to have the ability to use DES and DEDS as sole carbon and energy source. It was the intention to establish the primary cleavage or oxidative steps in the breakdown of these compounds.

1.5.3 The microbiology of methane sulphonic acid

An isolation methodology was to be established that would determine if MSA was degraded by bacteria. If so, the intention was to find which sulphur-containing compounds are the major products of degradation, enabling MSA to be fully included in the biogeochemical sulphur cycle.

CHAPTER TWO:
MATERIALS AND METHODS

2.1 Type Cultures.

The suppliers of the type cultures used in this work are listed in table 2.1.1.

The "Harfoot" Culture Collection were bacteria isolated by Dr C.Harfoot from the sources listed in table 2.1.2

Species	Strain	Source
Pseudomonas paucimobilis	-	N.T.T.C.
Pseudomonas aeruginosa	KF	K.P.Flint [1]
Pseudomonas fluorescens	KF	K.P.Flint [1]
Methylobacterium extorquens	AM1	P.Large [4]
Methylobacillus glycogenes	-	P.Green [2]
Methylophilus methylotrophus	-	P.Green [2]
Methylococcus methanolica	-	L.J.Zatman [3]
Methylococcus methylovora	-	L.J.Zatman [3]
Pseudomonas Sp.	MS	P.Large [4]
Pseudomonas Sp.	3A2	L.J.Zatman [3]
Methylotroph	5B1	L.J.Zatman [3]
Methylotroph	3A1	L.J.Zatman [3]
Methylotroph	S2A1	L.J.Zatman [3]
Bacillus Sp.	PM6	L.J.Zatman [3]
Methylotroph	5H2	L.J.Zatman [3]
Methylosinus trichosporium	OB3b	D.L.Cardy [1]
Methylococcus capsulatus	Bath	D.L.Cardy [1]
Methanotroph	IR1	R.Lees [1]
Methanotroph	DR1	R.Lees [1]
Thiobacillus versutus	A2	A.Wood [1]
Thiobacillus thyasiris	-	A.Wood [1]
Thiobacillus thioparus	E6	N.Smith [1]
Rhodococcus sp.	TTD-1	J.Green [1]

Table 2.1.1 Type cultures and their suppliers.

1 Department of Biological Sciences, University of Warwick, Coventry, UK.

2 N.C.I.M.B. Ltd, Aberdeen, UK

3 Department of Microbiology, University of Reading, Reading, UK.

4 Department of Microbiology, University of Hull, Hull, UK

Isolate	Isolated On	Isolated from	Gram Stain	Shape	Arrangement	Dimensions µm
M1/5S	DMS	Tikitere 1	+	1	5	0.7 x 3.0
MVA	DMS	Erebus steam vent	+	2	5	0.8 x 5.0
MVA/2	DMS	Erebus steam vent	+	2	6	0.7 x 3.0
EV/1	DES	Erebus steam vent	+	3	7	0.7 x 5.0
MWD	DMS	White Island	+	1	6	0.7 x 3.0
MFC	DMS	Lake Fryxell oxycline	+	1	8	0.7 x 5.0
TV3	$S_2O_3^{2-}$	Erebus steam vent	+	3	9	0.4
E1/3	DES	Tikitere 1	+	2	10	0.3 x 1.0
MFCY	DMS	Lake Fryxell oxycline	+	2	10	0.7 x 5.0
E4	DES	Tikitere 4	+	2	10	0.4 x 3.0
M4/20	DMS	Tikitere 4	+	3	11	0.4 x 1.0
D4	DMDS	Tikitere 4	-	3	10	0.7 x 1.0
E1/3S	DES	Tikitere 1	-	4	12	0.7 x 1.0
E1/3SY	DES	Tikitere 1	-	2	10	0.3 x 1.0
E1/2	DES	Tikitere 1	-	2	10	0.3 x 1.0
TF/1	$S_2O_3^{2-}$	Lake Fryxell oxycline	-	2	10	0.5 x 1.5
EC/1	DES	Erebus side crater	-	2	10	0.5 x 1.5
T1/B	$S_2O_3^{2-}$	Lake Fryxell oxycline	-	2	10	0.3 x 1.0

Table 2.1.2 The Harfoot culture collection

White Island is off the coast of New Zealand. Tiketere is an area with thermal vents in New Zealand. Tikitere 1 was a soil sample from under Leptospermum scoparium bushes, Tikitere 4 was a run off stream at approximately 30 °C with deposits of graphite and bacterial and algal growth. Mount Erebus is an active volcano on Ross Island, Antarctia. Samples were taken from near steam vents. Lake Fryxell is an antarctic lake showing thermal separation with depth.

Key: 1 = Slightly curved rod; 2 = Straight rod; 3 = Coccal rod; 4 = Coccus; 5 = Single or Vs; 6 = Singles, Vs or chains; 7 = Chains; 8 = Vs; 9 = Pairs, 4s or chains; 10 = Single; 11 = Pairs or Vs; 12 = Singles, pairs or clumps.

2.2 Media

2.2.1 Media supplements

The following supplements to minimal media were used throughout the work.

2.2.1.1 Vitamin solution

Vitamin solution (Kanagawa *et al.*, 1982) was made up to a final volume of 1000 ml at pH 7.0. It contained:

Thiamine hydrochloride	10 mg
Nicotinic acid	20 mg
Pryrodoxine hydrochloride	20 mg
p-aminobenzoic acid	10 mg
Riboflavin	20 mg
Calcium pantothenate	20 mg
Biotin	1 mg
Cyanocobalamin	1 mg

The solution was stored at 4 °C, protected from light.

2.2.1.2 Trace element solution (Modified Trudinger salts solution)

Trace element solution (Tuovinen and Kelly, 1973) was made up to a final volume of 1000 ml at pH 6.0. It contained:

Disodium EDTA	50 g
NaOH	10.2 g
$ZnSO_4.7H_2O$	11 g
$CaCl_2$	5 g
$MnCl_2.4_{H2}O$	2.5 g
$CoCl_2.6H_2O$	0.5 g
$(NH_4)_6Mo_7O_{24}$	0.5 g
$FeSO_4.7H_2O$	5 g
$CuSO_4.5H_2O$	0.2 g

The solution was stored at room temperature, protected from light.

2.2.2 Media for the growth of thiobacilli

2.2.2.1 Medium for the growth of *Thiobacillus versutus*

This medium was originally described by Taylor and Hoare (1969) and adapted by Wood and Kelly (1977). It contained, in 1000 ml distilled water:

$Na_2HPO_4.2H_2O$	7.9 g
KH_2PO_4	1.5 g
NH_4Cl	0.4 g
$MgSO_4.7H_2O$	0.1 g
$Na_2SO_3.5H_2O$	5.0 g
NaOH	0.46 g
Trace metal solution	10.0 ml
Phenol red	3.0 mg

Solid media also contained Difco 'Bacto' agar (15 g l^{-1}). The medium was autoclaved at 10 psi for 10 minutes at 115.5°C. Magnesium chloride and the trace element solution were autoclaved separately and added on cooling.

2.2.2.2 Medium for the growth of acidophillic thiobacilli (TvAM)

The medium was adapted from the *T. versutus* medium above by the omission of NaOH and Na_2HPO_4. It contained, in 1000 ml distilled water:

KH_2PO_4	30.0 g
NH_4Cl	0.4 g
$MgSO_4.7H_2O$	0.1 g
$Na_2SO_3.5H_2O$	5.0 g
Trace metal solution	10.0 ml
Saturated aqueous bromo-cresol purple	1.0 ml

Solid media also contained Difco 'Bacto' agar (15 g). The medium was autoclaved at 10 psi for 10 minutes at 115.5 °C. Magnesium chloride and the trace element solution were autoclaved separately and added after cooling.

2.2.2.3 Medium for the growth of *Thiobacillus thioparus* and CS_2 users [Basal Medium C (BMC)]

The medium, (adapted from that of Kanagawa and Kelly, 1986), contained, in 1000ml distilled water:

K_2HPO_4	2.0 g
KH_2PO_4	2.0 g
NH_4Cl	0.4 g
Na_2CO_3	0.4 g
$MgCl_2.6H_2O$	0.4 g
Trace metal solution	2 ml
Vitamin solution	5 ml

Solid media also contained Difco 'Bacto' agar (15 g l^{-1}). The medium was autoclaved at 10 psi for 10 minutes at 121 °C. Magnesium chloride and trace element solutions were autoclaved separately. The desired filter-sterilised substrates were added after autoclaving, except in the case

of sodium thiosulphate (routinely added at 0.5 g l^{-1}) which was added before autoclaving.

2.2.3 Medium for the growth of the Harfoot culture collection and CS_2 users [Basal Medium B (BMB)]

The medium was adapted from that used by Kanagawa and Kelly (1987). It contained, in 1000 ml distilled water:

K_2HPO_4	2.0 g
KH_2PO_4	2.0 g
NH_4Cl	0.4 g
Na_2CO_3	0.4 g
$MgCl_2.6H_2O$	0.4 g
Yeast Extract	0.2 g
Trace metal solution	2 ml
Vitamin solution	5 ml

Solid media also contained pre-washed Difco 'Bacto' agar (15 g). The medium was autoclaved at 10 psi for 10 minutes at 115.5 °C. Magnesium chloride and trace element solutions were autoclaved separately. Suitable filter sterilised substrates were added after autoclaving.

2.2.4 Media for the growth of Pseudomonas species.

2.2.4.1 Medium for the growth of *Pseudomonas* strain MS.

The medium, adapted from Kung and Wagner (1970) contained in 1000 ml:

K_2HPO_4	1.55 g
NaH_2PO_4	0.57 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4.7H_2O$	0.2 g
Trace metal solution	0.2 ml
Methylamine HCl	5.0 g

Solid media contained 20 g l^{-1} Difco "Bacto" agar in addition to the above. The medium was autoclaved at 126 °C and 15 psi for 15 minutes. The phosphate solutions were autoclaved separately

2.2.4.2 Medium for the growth of *Pseudomonas paucimobilis*.

This medium was adapted from that of Shimamato and Berk (1979), in that vitamin and trace element concentrations are changed. The medium contained, in 1000 ml distilled water:

K_2HPO_4	4.5 g
KH_2PO_4	1.0 g
$MgCl_2.6H_2O$	0.5 g
$MgSO_4.7H_2O$	0.5 g
NH_4Cl	0.5 g
NaCl	0.5 g
Sodium pyruvate	5.0 g
Trace metal solution	2 ml
Vitamin solution	5 ml

Solid media also contained 15 g l^{-1} Difco "Bacto" agar in addition to the above. The medium was autoclaved at 126 °C and 15 psi for 15 minutes. The phosphate solutions were

autoclaved separately and added after cooling.

2.2.5 Medium for the Oxidative/Fermentative (O/F) test (Hugh and Leifson's Medium)

Cultures in this medium (Hugh and Leifson 1953) were used to assess the production of acid during growth on carbohydrate under aerobic and anaerobic conditions. The medium contained, in 1000 ml:

Peptone	2.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Aqueous bromothymol blue (1% w/v)	3 ml
Difco 'Bacto' agar	3.0 g

The medium was adjusted to pH 7.1 before the addition of bromothymol blue. Sterilisation (at 126 °C and 15 psi for 15 minutes) was achieved by autoclaving in boiling tubes in which the depth of the medium was approximately 4 cm. Filter-sterile carbohydrate solution was added to 1% (w/v) on cooling of the medium to *c.* 45 °C. Duplicate tubes were seeded with culture while the agar was still molten, and one tube immediately covered with 4 ml sterile, molten petroleum jelly. The tubes were incubated at an appropriate temperature for up to two months.

2.2.6 Media for the growth of methylotrophs.

2.2.6.1 Medium for the isolation and growth of methylotrophs M1 to M9 [Minimal Medium E minus sulphate (MinE-S)

This medium was an adaptation of the medium of Owens and Keddie (1969). Type culture collection bacteria were also grown on MSA on this medium, unless otherwise stated. It contained, in 1000 ml distilled water:

K_2HPO_4	1.2 g
KH_2PO_4	0.624 g
$CaCl_2.6H_2O$	0.05 g
$MgCl_2.6H_2O$	0.165 g
NH_4Cl	0.5 g
Trace metal solution	2 ml
Vitamin solution	5 ml

Solid media were prepared by the addition of 15 g l^{-1} Difco 'Bacto' agar. The medium was autoclaved at 15 psi and 126 °C for 15 minutes. The phosphate was autoclaved separately. Substrates were routinely added as filter-sterile solutions to yield a final concentration of 15 mM, unless otherwise stated.

2.2.6.2 General medium for the growth of facultative methylotrophs on non-sulphur-containing substrates [Minimal Medium E (MinE)]

It was adapted from the medium used by Owens and Keddie (1969), subsequently used by Zatman (Colby and Zatman, 1972) for methylotroph isolation. The trace element and vitamin solutions used differ from the original medium. MinE contained, per litre of distilled water:

K_2HPO_4	1.2 g
KH_2PO_4	0.624 g
$CaCl_2.6H_2O$	0.05 g
$MgSO_4.7H_2O$	0.165 g
$(NH_4)_2SO_4$	0.5 g
Trace metal solution	2 ml
Vitamin solution	5 ml

Solid media were prepared by the addition of 15 g l^{-1} Difco 'Bacto' agar. The medium was autoclaved at 15 psi and 126 °C for 15 minutes. The phosphate was autoclaved separately. Substrates were routinely added as filter-sterile solutions to yield a final concentration of 15 mM, unless otherwise stated.

2.2.6.3 Medium for the growth of methanotrophs [nitrate minimal salts medium (NMS)]

This medium was described by Dalton and Whittenbury (1976), and contained, in 1000 ml:

K_2HPO_4	1.55 g
NaH_2PO_4	0.57 g
$MgSO_4.7H_2O$	10.0 g
KNO_3	10.0 g
$CaCl_2$	2.0 g
Trace Elements	10 ml

Solid media were prepared by the addition of 15 g l^{-1} Difco 'Bacto' agar. The phosphate solutions were autoclaved separately and added after cooling. The medium was

autoclaved at 15 psi and 126 °C for 15 minutes.

2.2.6.4 Medium to test for the growth of organism M2 on thiosulphate [Minimal medium F minus sulphate (MinF-S)]

This medium was an adaptation of minimal medium E (Owens and Keddie, 1969). It contained:

$Na_2HPO_4.2H_2O$	7.9 g
KH_2PO_4	1.5 g
$CaCl_2.6H_2O$	0.05 g
$MgCl_2.6H_2O$	0.165 g
NH_4Cl	0.5 g
Trace metal solution	2 ml
Vitamin solution	5 ml
Saturated aqueous bromo-cresol purple	0.25 ml
Sodium hydrogen carbonate (0.25M)	20 ml
Distilled water	972.75 ml

The medium was autoclaved at 15 psi and 126 °C for 15 minutes. The phosphate was autoclaved separately. Sodium hydrogen carbonate was added filter-sterilized after cooling.

2.2.7 Complex Buffers

The buffer (M2 buffer) used in experiments with methylotroph M2 (eg preparation of cell-free extracts, harvesting cells) was essentially minimal medium E without carbon source, vitamin or trace element solutions. The concentration of magnesium salt was doubled. "M2 buffer" contained, in 1000 ml:

K_2HPO_4	1.2 g
KH_2PO_4	0.624 g
$CaCl_2.6H_2O$	0.05 g
$MgSO_4.7H_2O$	0.33 g
$(NH_4)_2SO_4$	0.5 g

The medium was autoclaved at 15 psi and 126 °C for 15 minutes. The phosphates were autoclaved separately.

2.3 Isolation of Bacteria Using CDS as Sole Carbon and Energy Source

Soil and surface waters collected at Courtaulds Sulphur Chemicals Ltd. (Stretford, Manchester) were added to a series of seven Quickfit Erlenmeyer flasks containing minimal medium B, and gently shaken at 30 °C. Weekly, for thirteen weeks, carbon disulphide was added to provide a nominal concentration of 3 mM. Subsequently, the intervals, to a maximum concentration of 10 mM. Each week, the contents of the flasks were allowed to settle for two hours and 50 ml of the culture fluid was replaced with fresh medium.

After eight months, samples were taken from each flask and streaked on to a minimal agar medium in glass petri dishes. A carbon or energy source was supplied by incubating the dishes in a gas jar containing sufficient concentration was increased by 1 mM at two week CS_2 to provide the medium with 4 mM substrate. Colonies from these plates were transferred to Subasealed universal bottles containing 4 mM CS_2 in 5 ml minimal medium B. Any culture showing turbidity and consumption of CS_2 (as measured by headspace gas

chromatography) was retsreaked on to agar medium.

2.4 Isolation of microaerophillic *Thiobacillus*-type organisms

The isolation of microaerophiles from the Harfoot cultures was adapted from a method outlined by Nelson and Jannasch (1983; Nelson, 1989). The Harfoot cultures were inoculated into separate Erlenmayer flasks containing 50 ml MinB, with 5 mM DES as sole source of carbon and energy. When the cultures had reached mid-log phase, 10 ml of the medium was used to inoculate boiling tubes containing *Thiobacillus versutus* medium (with 0.2% (w/v) agar), held at a temperature of 45 °C. The agar and bacteria were thoroughly mixed by inverting the boiling tube, and allowed to gel. The tubes were then incubated at 30 °C.

After two weeks incubation, a discrete band of growth could be seen below the surface of the agar. The outside of the boiling tube was aseptically cut above and below the band of growth, and the agar pushed out of the glass into a sterile petri dish. The agar was then cut up into cubes of approximately $1mm^3$ using a sterile scalpel. The cubes of agar were used to inoculate Subasealed Quickfit flasks containing 50 ml *T. versutus* medium. The concentration of oxygen in the flasks was varied from 1 to 15% by flushing them with sterile oxygen-free nitrogen before an applicable amount of sterile oxygen was added. Those flasks which appeared turbid after two weeks incubation at 30 °C were streaked onto 1.5% agar in *T. versutus* medium and 1.5% agar

in MinB (with 5 mM DES). These plates were incubated under reduced oxygen tension (achieved in a similar way to above) in sealed jars at 30 °C. Any single colonies were picked and streaked onto slopes and incubated, again at an appropriate oxygen concentration.

2.5 Isolation of bacteria using methane sulphonate as sole carbon and energy source

Soil (100 g) from the University of Warwick campus was used to inoculate a one litre fermenter. Initially this was run as a batch culture (30 °C, stirring 500 rpm, aeration at 600 ml/min) in MinE-S containing 10 mM MSA and 20 mM methylammonium chloride (MMA). After two weeks, the vessel was fed intermittently (one hour on, two hours off) with medium containing 7 mM MSA and 3 mM MMA, at a dilution rate of 0.03 h^{-1}. Samples were taken after 5 weeks, streaked on to MinE-S containing 15 mM MSA alone, and incubated at 30 °C for five days.

2.6 Culture preservation

2.6.1 Short term preservation

The methylotroph M2 was maintained on solid media by sub-culturing at bi-monthly intervals onto agar slopes prepared from the MinE, with 15 mM methylamine (MMA) as sole carbon and energy source. Type cultures were also maintained this way, using suitable media and carbon sources. Cultures were streaked onto agar plates and then subcultured through liquid media before restreaking onto fresh agar slopes.

2.6.2 Long term preservation

The long term storage of M2 was attempted by freezing cells suspensions at -70 °C in a variety of media. Glass beads (2-3 mm in diameter) were washed in detergent, followed by washes in 1% (v/v) hydrochloric acid, tap water and distiled water. The beads were placed in bijoux bottles so that they filled approximately half the bottle. The bijoux were then autoclaved at 15 psi and 126 °C for 15 minutes. Cells were washed twice in sterile M2 buffer and resuspended in either glycerol (15% v/v), skimmed milk (10% w/v) or M2 buffer (all sterile solutions). These suspensions were used to thoroughly wet duplicate aliquots of glass beads. Excess liquid was removed before freezing at -70 °C.

Recovery of these frozen stocks was achieved by aseptically removing one of the glass beads and rubbing the bead over a suitable solid medium (MinE with 15 mM MMA, in the case of M2). The remaining beads were immediately returned to the freezer.

2.6.3 Culture purity checks

The purity of strain M2 was checked microscopically, and by plating out dilutions in M2 buffer onto nutrient agar (NA), MSA agar and formate agar. The criteria for purity were uniformity of colony morphology on MSA agar and formate agar and absence of growth on NA. The most common contaminants were gram negative rods, possibly of an enteric type, which were easily detectable by their growth on NA.

2.7 Growth in batch and continuous culture

2.7.1 Batch culture of bacteria growing on organic sulphides

The Harfoot collection of bacteria were routinely grown in sterile 250ml "Quickfit" conical flasks, with 50ml of basal medium B. Substrates such as diethyl sulphide and dimethyl sulphide were injected into the flasks after they had been sealed with Subaseals. Since rubber Subaseals absorb organic sulphides, new Subaseals were used for every flask, and during growth experiments, a control of uninoculated subasealed medium with substrate was used. Bacteria growing on carbon disulphide were treated in a similar manner, except that the media used was basal medium C.

Solid media were prepared without substrate. Petri dishes were placed in an anaerobic jar, sealed, but no air removed. Volatile organic sulphide substrates were then injected through the rubber seal of the jar so that the concentration of sulphide was 10 mmol per litre of air. A petri dish containing dried silica gel was also placed in the jar to reduce humidity. Concentration of organic sulphides were monitored throughout the incubation by headspace gas chromatography (see below), and adjusted if necessary.

Since the above method led to some leakage of sulphide from the jar, it was thought to be unsuitable for use with cultures growing on the toxic compound carbon disulphide. Instead solid medium was poured into 250 ml Quickfit flasks and allowed to set so that a vertical agar slab filled half the flask. Cultures could then be streaked onto the agar and

the flask was Subasealed. Carbon disulphide was then injected into the flask to a known concentration. As the agar was incubated vertically, then the problem of water accumulating on the surface of the medium was reduced, compared to initial atempts with agar filling the base of a 250 ml flask.

Cultures using DES and CDS were examined for microaerophillic or anaerobic growth by introducing 0.2 ml of a suspension of the culture into a Subasealed test-tube containing 20ml of a suitable medium. This medium contained 1.5% (w/v) agar and was maintained at 45 °C during inoculation. Volatile substrates were then introduced into the medium and mixed by inversion of the test tube.

2.7.2 Batch culture of methylotroph M2

The methylotrophic strain M2 was grown in 50 ml MinE or MinE-S (depending on carbon-source) at 30 °C with orbital shaking at 150 rpm. When growing on MSA, 10 µl sterile saturated aqueous bromothymol purple was added to the medium. When the pH in the flask had dropped sufficiently to turn the indicator yellow, the medium was neutralised by the addition of 1 or 2 M NaOH. This prevented cultures from becoming pH limited, but this technique was not used during the preparation of growth curves.

2.7.3 Continuous culture of methylotroph M2

Cultures of the methylotroph M2 were routinely grown in chemostats to provide biomass for enzyme assays and oxygen

electrode experiments. Chemostats were constructed from 1000 ml or 750 ml water jacketed vessels (Cutforths, Birmingham) with LH 100 series (LH Fermentation Ltd, Maidenhead, England) stainless steel lids. The pH of the media was controlled by automatic titration with 2 M HCl or 2 M NaOH. Stirring at 750 rpm was by means of magnetic paddle stirrers and air supplied at 1 ml (ml culture)$^{-1}$ minute^{-1}. A constant temperature of 30 °C was maintained by means of circulation of thermostated water (Churchill Ltd). Peristaltic pumps (Watson-Marlow Ltd) provided fresh medium to the chemostat. Spent medium was expelled from the vessel into a waste pot.

The inoculum for chemostats was prepared as follows. Bacteria were removed from a slope and inoculated into 50ml of MinE (with 15 mM MMA as sole source of carbon and energy) in an Erlenmeyer flask. Once the culture had reached an OD_{440} of 0.8, it was used as an inoculum for a 2 litre Erlenmeyer flask containing 400 ml MinE and 15mM of the carbon source to be used as the limiting nutrient in the chemostat. The 400 ml culture was aseptically introduced into the chemostat once the culture had reached an OD_{440} of 0.8. The chemostat was then filled to its operating volume with fresh medium. Medium pumping commenced once the culture had reached log phase again, routinely at a dilution rate of 0.005 h^{-1} or below.

2.7.4 Continuous culture of Thiobacillus versutus

Chemostats were constructed from a 1000 ml jacketed vessel (Cutforths, Birmingham) with an LH 100 series (LH

Fermentation Ltd, Maidenhead, England) stainless steel lid. The pH of the media was controlled by automatic titration with 2 M NaOH. Stirring at 750 rpm was by means of magnetic stirrers and air supplied at 1 ml (ml culture)$^{-1}$ minute^{-1}. Peristaltic pumps (Watson-Marlow Ltd) provided fresh medium to the chemostat. A constant temperature of 30 °C was maintained by means of circulation of thermostated water (Churchill Ltd). Spent medium was expelled from the vessel into a waste pot.

The inoculum for chemostats was prepared as follows. Bacteria were removed from a slope and inoculated into 25 ml TvM in an Erlenmeyer flask. Once the culture had reached an OD_{440} of 0.2, it was used as an inoculum for a 2 litre Erlenmeyer flask containing 200 ml TvM, and the culture allowed to grow up as before. The 200ml culture was aseptically introduced into the chemostat. The chemostat was then filled to its operating volume with fresh medium. Medium pumping commenced once the culture had reached log phase again, routinely at a dilution rate of 0.005 h^{-1} or below.

2.7.5 Batch culture of other bacterial strains.

Type culture bacteria were grown in 25 ml of an appropriate medium in a 250 ml Erlenmeyer flask and incubated at 30 °C with orbital shaking. *Methylosinus trichosporium*, *Methylococcus capsulatus*, Methanotroph IR1 and Methanotroph DR1 were supplied as ready-grown cultures by V.Lees and

D.L.Cardy, University of Warwick.

2.8 Identification of bacterial cultures

2.8.1 Gram-negative rods

Bacteria such as M2 were putatively identified using the API20B and API20E multitest systems.

The API20E system tested for: gelatin hydrolysis, production of nitrate from nitrite, ß-galactosidase, the production of acid from a suspension of cells in presence of the following compounds - saccharose, arabinose, mannitol, fructose, glucose, maltose, starch, rhamnose, galactose, mannose, sorbitol, inositol, melibiose, amygdalin and glycerol. Also the system detected the presence of urease, indole, the ability to form H_2S, acetoin, cytochrome oxidase, arginine, dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, catalase, oxidase and motility. The Simmon's citrate test was also included.

2.8.2 Coryneform bacteria

The Harfoot cultures MVAe, MVAm, MVA/2, EV1, E1/3, MWD, M4/20 and M1/5S were subjected to 25 biochemical and physiogical tests devised by Sieler and co-workers (Sieler *et al.*, 1977; Sieler *et al.*, 1980; Sieler, 1983). The tests were:

1) The utilization of pyruvate, DL-lactate, propionate, 5-amino valerate, DL-malate, succinate, citrate, 4-hydroxybenzoate, L-aspartate, glycine, L-arginine, L-

histidine, D-galactose, D-xylose, D-ribose, L-arabinose, xylitol and D-mannitol as sole sources of carbon and energy, supplied at 0.1% (w/v).

2) Hydrolysis of milk, tyrosine and xanthine

2) Other conventional tests (O/F test, motility, catalase, oxidase, Vosges Proskauer, indole, urease, ONPG, liquifaction of gelatin, acid from the metabolism of arabinose, mannitol, fructose, glucose, maltose, sucrose and xylose)

These tests were used to generate a simple matching coefficient. An operator within the Lotus 1-2-3 program for IBM compatible personal computers was used to compare the results to those clusters suggested by Sieler.

2.9 Analytical methods

2.9.1 The chemical estimation of formate

Formate concentrations in culture supernatants were determined using the method of Lang and Lang (1972). The sample was diluted with M2 buffer so that the formate concentration was in the range of 1-5 mM. The diluted sample (0.5 ml) was then mixed with 1 ml of a 0.5% citric acid and 10% acetamide solution (both w/v, in isopropanol). Sodium acetate was then mixed with the above, as an aqueous 30% solution. Acetic anhydride (2 ml) initiated the formation of a green colouration in the presence of formate. The optical density of the mixture was read at 515 nm after 2 hours incubation at room temperature. Formate concentration was

calculated from a standard curve, obtained with 0-5 mM potassium formate.

2.9.2 The chemical estimation of formaldehyde.

The concentration of formaldehyde in culture supernatants and in solutions prepared from paraformaldehyde was determined using the method developed by Nash (1975). The "Nash reagent" was prepared by dissolving acetyl acetone (2 ml), acetic acid (3 ml) and ammonium acetate (150 g) in distilled water and made up to 1000 ml. The reagent (2 ml) was added to 0.5 ml of the sample to be tested and incubated for 45 minutes at 37 °C. This resulted in the formation of a yellow colour, which could be quantified by means of a spectrophotometer set at 412 nm. Concentration of formaldehyde in the sample was calculated by comparison against a standard curve of "Analar" formaldehyde and methanol solution (BDH).

2.9.3 The chemical estimation of sulphur

This method depends on the formation of an iron-sulphur complex, detectable spectrophotometrically at 470 nm.

The sample was diluted to the range of approximately 1-5 mM sulphur and made up to 5 ml with distilled water. To this was added 15 ml 0.1% (w/v) KCN (The KCN was made up in 380 ml acetone and 20 ml water.). After shaking the mixture was allowed to stand for 2 minutes before being made up to 25 ml with the acetone and water solution. A solution in acetone of 0.4% (w/v) ferric chloride was made up and added

in equal volumes to the mixture. Reading spectrophotometrically at 470 nm against a blank of 5 ml water treated in the same way as the sample gave a qualitative estimation of the presence of sulphur.

2.9.4 The chemical estimation of sulphate

The concentration of sulphate in a solution can be calculated by precipitating the ion as barium sulphate and measuring the residual soluble barium by atomic absorption spectrophotometry.

A culture sample was taken and centrifuged so that no whole cells remained. The supernatant was removed and used for the assay. The supernatant was diluted in 1% (v/v) HCl so that sulphate was in the concentration range of 50-175 mg l^{-1}, and was in a volume of 5 ml. An equal volume of barium chloride (1.5 gl^{-1}, in 1% v/v HCl) was mixed thoroughly with the sample. The precipitate of insoluble barium sulphate was allowed to settle overnight. Residual barium chloride in the clear solution was estimated using an atomic absorption spectrophotometer set at 553.6 nm, against a standard curve prepared from solutions of potassium hydrogen sulphate, diluted as before in 1% (v/v) HCl.

2.9.5 Gas chromatography

2.9.5.1 The determination of methanol.

Methanol concentrations in culture supernatants were determined by packed column gas chromatography. A Poropak Q column was used under the following conditions in a Pye

Unicam series 204 gas chromatograph: nitrogen, 30 ml minute^{-1}, air, 0.5 kg cm^{-2}, hydrogen 1.0 kg cm^{-2}, oven temperature 180 °C, injector temperature 200 °C, detector 250 °C. Peak detection was via a flame ionisation detector linked to a Hewlett-Packard computing integrator. The standard used was 5 µl of a 2 mM methanol solution.

2.9.5.2 The determination of organic sulphur compounds

The concentrations of sulphides and thiols were determined by packed column headspace gas chromatography. The column used was a 34 ft, 0.085 inch (i.d.) length of Teflon (FEP), packed with Chromasorb T, polyphenyl ether (12%) and H_3PO_4 (0.5%). The chromatograph used was a Pye Unicam series 204, linked to a Spectra Physics PU 8140 integrator. The column was maintained isothermally at 110 °C, with nitrogen carrier gas at 60 ml min^{-1}. Compounds were detected using a flame photometric detector. Headspace samples were analysed, (see example of chromatogram report, fig 2.9.5.2).

Sulphur dioxide standards were prepared by flushing a 100 ml Subasealed Erlenmeyer flask with pure SO_2 for 5 minutes. A sufficient amount of this gas was injected into a 250 ml Subasealed 250 ml Quickfit flask (containing 50 ml basal medium B) to yield SO_2 concentrations equivalent to between 1 and 3 mM in the liquid. Peak areas from injecting 100 µl headspace from these standards were assigned log values in the preparation of the standard curve. Thus by considering concentrations of gas in the headspace above the standards, the gas/liquid partition effect could be ignored in sample

flasks. Concentrations of the gas in the medium could then be deduced from headspace results. Hydrogen sulphide standards were prepared in a similar way.

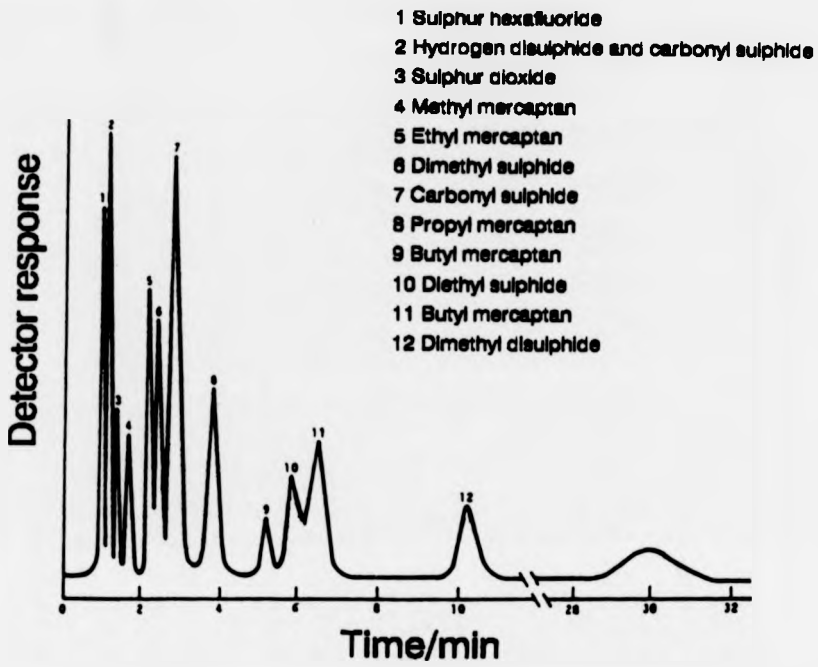


Figure 2.9.5.2 Retention times of organosulphur compounds in a 30 foot teflon column, detection via a GC-FPD.

Carbon Disulphide standards were prepared by adding sufficient pure liquid CS_2 to a 250 ml Subasealed Quickfit flask (containing 50 ml basal medium B) to yield CS_2 concentrations equivalent to between 1 and 3 mM in the medium. Peak areas obtained from injecting 100 µl headspace of these standards were assigned log values in the preparation of a standard curve.

2.9.6 Nuclear magnetic resonance spectroscopy

The proton nuclear magnetic spectra of culture supernatants was ascertained with the help of Dr O. Howarth, Department of Chemistry, University of Warwick.

2.9.7 Measurement of biological oxidation in the oxygen electrode.

Oxidation studies were performed by measuring changes in dissolved oxygen in a Clarke-type oxygen electrode (Rank Brothers, Bottisham, Kent), detected via a chart recorder (Gallenkamp, UK) The perspex cell consisted of a base in which two electrodes are separated by 1 ml saturated aqueous KCl. A teflon membrane, approximately 2 cm^2 was placed over the top of this so that no air bubbles were trapped underneath. Half of the KCl solution was then removed so that the teflon fitted closely around the protruding electrodes. The top half of the cell was then screwed down tightly over the membrane, providing a 5 ml well in which the reaction mixture was placed. The mixture was isolated from the atmosphere by means of a plug with a narrow bore hole in. This hole allowed insignificant gaseous exchange, but did allow the insertion of a needle into the reaction mixture.

The temperature of the cell was maintained at 30 °C (45 °C in the case of *Methylococcus capsulatus*.) by means of thermostatted water circulating through a jacket surrounding the cell. Once the temperature in the cell was constant, 3 ml of distilled water was introduced into the cell, and

the chart recorder was adjusted to full scale deflection. The amount of oxygen in air-saturated water was taken to be 245 nmoles ml^{-1}. The chart recorder was zeroed by adding a few crystals of sodium dithionite to the water, and then sealing the cell with the plug, expelling all of the air from above the solution.

2.9.8 Protein Profiles of Whole Cells and Cell-free Extracts by Polyacrylamide Gel Electrophoresis (PAGE).

2.9.8.1 Sample Preparation.

Bacterial cells were harvested, washed twice in a suitable buffer (M2 buffer for most methylotrophs) and resuspended in a volume of sample preparation buffer equal to the volume of the pellet. The sample preparation buffer contained: 0.5 M tris-HCl (2.0 ml, pH 6.8), Glycerol (1.6 ml), 10% aqueous sodium dodecyl sulphate (3.2 ml), ß-mercaptoethanol (0.8 ml), 0.1% aqueous bromophenol blue (0.4 ml). This mixture was placed in a boiling water bath for ten minutes. A clear supernatant was prepared by centrifugation in a Microfuge for 5 minutes. Those samples that yielded a supernatant that appeared to be opaque and viscous were clarified by passing through glass wool. The protein concentration of the supernatant was assessed before the sample was used for PAGE.

After use, the samples were stored at -20 °C. If they were to be used subsequently, the samples were boiled again for 5 minutes.

2.9.8.2 Acrylamide gel composition.

Gels were poured between vertical glass plates held 4 mm apart by perspex formers (BRL Inc, Rockville, USA). Plates and formers had previously been washed with a mild detergent, 1% HCl, distilled water, ethanol and acetone.

A resolving gel of 10.5% polyacrylamide routinely contained 30% acrylamide (14 ml), distilled water (11 ml) and tris-HCL pH 8.8 (5 ml, containing 10% sodium dodecyl sulphate). The curing agents for polymerisation were 0.28% ammonium persulphate (10 ml) and TEMED (23 µl). The gel was allowed to set vertically for 1½ hours with the exposed surface covered by 1 or 2 ml of distilled water-saturated butan-2-ol.

Gradient resolving gels contained 20 ml of 10.5% polyacrylamide (as above), and 20 ml of 15% polyacrylamide, containing 30% acrylamide (20 ml), distilled water (5 ml), tris-HCL pH 8.8 (5 ml), 0.28% ammonium persulphate (10 ml) and TEMED (23 µl). The different solutions were mixed (immediately after addition of curing agents) via a gradient maker. The gel was allowed to set vertically for 1½ hours with the exposed surface covered by 1 or 2 ml of distilled water-saturated butan-2-ol.

The stacking gel for both gradient and single percentage gels was prepared by mixing the following: 6 ml acrylamide (30%), 19 ml distilled water, 5 ml tris-HCl (0.5 M, pH 6.8 containing 10% sodium dodecyl sulphate), 10 ml ammonium persulphate (0.28%) and 23 µl TEMED. A stacking gel of

approximately 5 cm depth was poured onto the resolving gel after the butan-1-ol had been removed by repeated washes with distilled water. A comb was inserted into the stacking gel before polymerisation had occurred, to make wells of approximately 200 µl in volume,. The gel was allowed to set for ½-1 hour. Unpolymerised acrylamide was removed from the wells by washing with distilled water.

2.9.8.3 Electrophoresis

The prepared gel was clamped into a gel rig (BRL Inc, Rockville, USA) and 500 ml of running buffer poured into each of the reservoirs. Running buffer contained (in 1500 ml distilled water) 4.5 g tris base, 21.6 g glycine and 1.5 g sodium dodecyl sulphate. Samples of a suitable protein concentration and content were loaded into the wells through the running buffer. Protein molecular weight markers (Pharmacia) were loaded alongside the samples in separate wells. A current of 4-10 mA was supplied by a Shandon Vokam 400 power pack (Shandon Vokam, Runcorn, England), and the proteins left to resolve overnight.

2.9.8.4 Protein visualisation

The resolving gel was removed from the glass plates and separated from the stacking gel. The proteins were stained by immersing the gel in a solution containing 40% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.046% (w/v) Coomasie blue R. The gel was left at room temperature for 2-8 hours. Unbound stain was removed by transferring the gel to a solution of 5:1:5 (v/v) methanol, glacial acetic acid

and water. The destining process was repeated until the protein bands were clearly defined.

2.9.9 Carbon Analysis

The total carbon content of cells and culture supernatants were estimated using a Beckman Total Carbon Analyser (Model 915B, Beckman Instruments Inc., California, U.S.A.). Solutions of known carbon concentration (0-100 mg l^{-1}) were prepared using potassium hydrogen phthalate and CO_2-free water. A standard curve from this data was then used to estimate carbon concentration in samples.

2.9.10 The incorporation of ^{14}C-labelled C_1-compounds into a chemostat limited by MSA

The incorporation of ^{14}C-labelled compounds into cells of M2 was performed in batch and continuous culture. Batch cultures were of standard media, except that a small quantity of carbon source was replaced with a labelled one, so that the activity of the entire medium was about 20,000 cpm ml^{-1}.

Chemostats at a steady state were supplied with 15 mM MSA at a dilution rate of 0.08 hr^{-1}. The medium supplying the chemostat was supplemented with C_1-substrates (methanol, formaldehyde and formate) so that the total carbon in the medium entering the chemostat was 16 mM. A small proportion of the additional substrate was ^{14}C-labelled.

Samples from either batch or continuous cultures were spun down in a microfuge as 1.5 or 2 ml aliquots, in plastic

screw-top Eppendorf tubes. The 0.5 ml of the supernatant was then removed and placed in a scintillation vial, to which 100 µl of 6 M phosphoric acid had already been added. These vials were left overnight in a fume hood, 10 ml scintillation fluid added and the radioactivity due to non-volatile compounds counted. Samples were also taken directly from the culture an applied in 0.5 ml aliquots to 2.5 cm diameter nitrocellulose filters (pore size 0.2 µm, Whatman Ltd, UK). These filters were washed twice with M2 buffer, under suction, and placed in vials. Counting took place immediately, using a Beckman LS7000 liquid scintillation spectrometer after the addition of "Optiphase safe" scintillation fluid.

2.9.11 Determination of antibiotic sensitivity.

Cultures of M2 were grown to mid-log phase on 30 mM MMA. Aliquots of these cultures (100 µl) were spread onto MinE agar plates containing 15 mM MSA or 15 mM formate. Mastrings (numbers M11, M12, and M43, Mast Laboratories, Bootle, UK), were laid on top of separate plates of each carbon source and pressed lightly into the medium. The plates were incubated at 30 °C until a lawn of bacteria appeared.

2.9.12 The isolation of DNA from bacteria

2.9.12.1 Extraction without cesium chloride

This method was developed by A.P. Wood (King's College, University of London), for the extraction of DNA from Gram negative sulphur bacteria.

Cells were harvested and resuspended in a volume of saline EDTA (0.15 M NaCl, 0.1 M sodium EDTA, pH 8.0) equal in volume to the cell pellet. For every 40 mg dry weight of cells, 1 ml 0.03 M NaOH was added. The cells lysed during incubation at room temperature for 3 minutes. The cell lysate solution was treated with 3.3 ml SDS saline EDTA (2.5 % (w/v) SDS 0.15 M NaCl, 0.1 M sodium EDTA, pH 7.0). RNA was removed by the action of 6 µl ribonuclease solution (1 mg ml^{-1} crystalline RNase, 10 mM TRIS-HCl, 15 mM NaCl, pH 7.5), left to incubate at 60 °C for 30 minutes. The protein content was also reduced by the addition of 12 µl of Proteinase K (200 mg ml^{-1}, aqueous). The cell lysate solution was incubated with the enzyme for 15 minutes at 37 °C.

The majority of unwanted cell debris was removed by separation across a water/alcohol interface. The volume of the solution was increased by a quarter with the addition of 5 M NaCl, and an equal volume of a chloroform:isoamylalcohol (24:1) solution was added to this. After 5 minutes at room temperature, cell debris was spun down at 5000 g for 5 minutes. The clear lysate now contained a mixture of macromolecules. Contaminated DNA was precipitated from the lysate by the addition of one tenth volume of sodium acetate (1 M), followed by 2 volumes of 95% ethanol at -20 °C. The lysate was stored at -20 °C overnight.

The solution was spun down at 5000 g for 5 minutes to remove the DNA precipitate. The pellet was resuspended in 0.8 ml one tenth strength SSC (sodium citrate 4.41 g, sodium

chloride, 8.77 g in 800 ml distilled water). The volume of the DNA solution was increased by one tenth by a supplement of 1 M NaCl. An equal volume of isopropylalcohol at -20 °C caused re-precipitation of the DNA after incubation at -20 °C for 60 minutes. The DNA was removed by centrifugation at 5000 g for 5 minutes, and resuspended in a minimum volume of one hundredth strength SSC. It was stored at -20 °C.

2.9.12.2 Extraction using cesium chloride gradient centrifugation

Cells were harvested and pelleted by centrifugation at 10,000 g. A cell suspension was made with 6 ml TE buffer. Lysis was accomplished by the addition of 1 ml 0.03 M NaOH. The cells lysed during incubation at room temperature for 3 minutes. The protein content was reduced by the addition of 12 µl of Proteinase K (200 mg ml^{-1}, aqueous). The cell lysate solution was incubated with the enzyme for 15 minutes at 37 °C. 3.25 ml SDS [10% (w/v)] was added, and the solution allowed to clear during an incubation at 37 °C, for between 1 and 3 hours.

The lysate was incubated at 60 °C with 4 ml sodium perchlorate for 15 minutes, with occasional stirring before it was transferred to a polypropylene "Oakridge" centrifuge tube. The volume of the solution was doubled with phenol:chloroform and mixed by inversion to give a milky solution. Aqueous and chloroform phases were separated by centrifugation at 35,000 g for 30 minutes. The lower chloroform phase was discarded and the upper phase removed

with the aid of a wide bore automatic pipette, to be placed in a fresh polypropylene tube. The phenol:chloroform extraction procedure was repeated up to five times.

The aqueous phase was supplemented with sodium chloride so that the final chloride concentration was 0.1 M. DNA was precipitated from this saline solution by the addition of two parts 99.9% ethanol, and stored at -20 °C. The DNA appears as a woolly mass, which can be removed with a thin glass rod or by centrifugation at 5000 g. The DNA is then washed in a series of clean universal bottles in 70% ethanol. The washed DNA was placed in a plastic universal, which was covered with a "Parafilm" membrane pierced with several holes, and vacuum desiccated for an hour.

The dry DNA was gently dissolved in 10 ml TE by gentle rotation overnight (~2 RPM). RNA was digested on the addition of 50 μl RNase solution (1 mg ml^{-1} crystalline RNase, 10 mM TRIS-HCl, 15 mM NaCl, pH 7.5), followed by incubation at 37 °C for 30 minutes. 10 ml of the RNA-free DNA solution was placed on 20 ml TE containing 33 g cesium chloride and 2 ml ethidium bromide (10 mg ml^{-1}), contained in a heat-sealed Oakridge tube. The DNA formed a band, visible under ultraviolet light, after centrifugation at 45,000 RPM in a Beckman VTI 50 rotor. The DNA was removed from the cesium chloride gradient by piercing the side of the Oakridge with a wide-bore needle, and drawing the luminescent band out. Ethidium bromide was removed from the DNA by extraction across an aqueous/isoamylalcohol interface, repeated until the pink colouration of the

bromide was completely removed from the aqueous phase. The DNA could be stored at 4 °C in the cesium chloride/TE solution, or dialysed against 5 l of SSC (first at room temperature, then at 4 °C), for storage at -20 °C.

2.9.12.3 Calculation of the %G+C of purified DNA

The thermal denaturation of DNA was measured spectrophotometrically to provide a measure of the percentage of guanisine and cytosine bases in the molecule compared to the total number of nucleotides.

2.9.13 Determination of dry weight of bacteria.

Cultures of M2 were grown in chemostat culture on MSA. Culture (800 ml) was harvested from the chemostat and resuspended in water to a total volume of 3 ml. Aliquots (0.5 ml) of the cell slurry were placed in pre-weighed borosilicate glass vials and allowed to dry at 100 °C. A portion of the slurry was diluted accurately, and the optical density measured at 440 nm. The glass vials were weighed daily until their weight had reached a constant minimum. The weight of the slurry was then compared to the optical density of the original solution of cells. The dry weight of the cells was expressed as mg per unit optical density. The process was repeated with cells at five different optical densities.

2.10 Enzyme assays

2.10.1 Introduction

All enzyme assays were performed on cell-free extracts of chemostat-grown cells. The chemostats were maintained at a dilution rate of 80% of the μmax for the particular carbon and energy source used. All enzyme assays were performed on cell-free extracts of chemostat-grown cells. The chemostats were maintained at a dilution rate of 80% of the μmax for the particular carbon and energy source used. Cell-free extracts were prepared by the French press method, or from Braun homogenates.

Cells were spun down from their media at 10,000 g at 4 °C and washed three times with M2 buffer. The cells were then centrifuged again, but resuspended in a minimum amount of buffer. The cell slurry was passed three times through a French press at 1000 lb $inch^{-2}$. Cell debris was removed from the crude extract by centrifugation at 50,000 g for one hour.

An alternative method of cell breakage, using the Braun homogeniser, was used in the preparation of the first extracts of M2. The cell slurry, in this case, is mixed with an equal volume of small glass beads (0.10-0.11 nm diameter) and placed in a borosilicate, thick-walled glass test tube, sealed by means of a screw cap. The beads and bacteria are shaken mechanically (with cooling) for 5½ minutes. The beads were removed from the crude extract by low speed centrifugation (4,000 g) for 30 minutes. This was followed

by centrifugation at 50,000 g for one hour, to produce a pellet containing cell debris and supernatant, as from the French press method. The Braun homogeniser was not used extensively during the work.

Spectrophotometric reactions were performed in a 1 cm light path silica cuvette, and changes in optical density were measured in a Pye Unicam PU8750 UV/VIS spectrophotometer, linked to an International Business Machines Personal System 2 Model 55 SX computer. The cuvette housing was kept at a constant 30°C by means of circulation of thermostatted water. Cell free extracts were stored on ice and used within 8 hours of preparation.

The activity of ^{14}C-labelled compounds was determined by dissolving samples in 10 ml 'Optiphase Safe' scintillation fluid and counting emissions in a Beckman LS7000 liquid scintillation counter.

2.10.2 Assay of hydroxypyruvate reductase

The formation of glycerate from lithium hydroxypyruvate was assessed by the decrease in optical density at 340 nm, caused by the formation of $NAD(P)^+$ according to the following reaction:

$$2\ \text{Hydroxypyruvate} + NAD(P)H + H^+ \Leftrightarrow \text{glycerate} + NAD(P)^+$$

The assay was performed using the method of Kelly and Wood (1984). The spectrophotometer was zeroed using 330 µl of buffer (0.1 M phosphate buffer, pH 7.0 or 0.1 M acetate buffer, pH 5.0) and enough water to make the final volume of

the reaction mixture equal to 1000 µl. Cofactor, in the form of NADH (1.3 mM) or NADPH (1.2 mM) was added and the optical density at 340 nm noted before the addition of a suitable quantity of cell-free extract. The cuvette was allowed to equilibrate for 2 minutes. Any endogenous NAD(P)H oxidation was followed for 2 minutes before the initiation of the assay with 50 µl lithium hydroxypyruvate (40 mM). Rates of reaction and the Michaelis constant for the enzyme was calculated using the molar extinction coefficient of NAD(P)H of 6200 $^{-1}cm^{-1}$.

2.10.3 Assay of hexulose phosphate synthase

The formation of hexulose-6-phosphate from formaldehyde and ribulose-5-phosphate was assessed by means of tracing ^{14}C-labelled substrates. The reaction is assumed to proceed in the following way:

Formaldehyde + Ribulose-5-phosphate <=> Hexulose-6-phosphate

The assay was performed using the method of Kelly and Wood (1984). The reaction mixture (total volume 0.4 ml) contained 50 mM phosphate buffer (pH 7.0), 12.5 mM $MgCl_2$, 5 mM ribose-5-phosphate, 5 or 10 mM ^{14}C-labelled formaldehyde (65,000 cpm μmol^{-1}), and 0.1 ml extract. Mixtures were prepared in triplicate. The extract was used to initiate the reaction and the mixture incubated at 30 °C, for 5, 10 or 20 minutes. The reaction was terminated by the addition of 1.5 ml of a formaldehyde (4% w/v), ethanol (90% v/v) and barium acetate (0.33% w/v) solution, followed by thorough mixing. The mixtures were then held on ice for 30 minutes, filtered

through 25 mm glass-fibre filters (Whatman GF/C) and washed with one iteration of 2 ml 2% (w/v) formaldehyde in ethanol and two of 1 ml ethanol. The filters were dried overnight at 60 °C. Fixed $H^{14}CHO$ was measured by scintillation counting of the whole filters.

2.10.4 Assay of ribulose 1,5-bisphosphate carboxylase (RUBISCO)

2.10.4.1 By the permeabilised whole cell method.

The rate of fixation of carbon dioxide by RUBISCO can be measured by examining the fixation of $^{14}CO_2$ into non-volatile, non-acid labile cellular constituents, as described by Kelly and Wood (1982). The method differs from that work in that cetyl trimethylammonium bromide (CTAB) was used as well as Triton X-100 (Leadbetter *et al.*, 1982). Both detergents were used at reduced concentration (from the work of M.Maclean (University of Warwick) on the RUBISCO of *Thiobacillus ferrooxidans*, unpublished data). Permeabilisation allows the substrates of RUBISCO access to the enzyme without the potentially harmful effects arising from cell breakage and high-speed centrifugation.

Cells were taken from a chemostat and placed in a four 4 ml glass "B & T" centrifuge tube, so that there was between 0.3 and 0.33 mg dry weight of cells per tube. The cells were then pelleted at full speed for ten minutes in a B & T microangle centrifuge (B & T Ltd, Essex, England). The supernatant was aspirated off and the pellets resuspended in either 0.2 ml Triton X-100 (5% aqueous solution) or 0.2 ml

CTAB (0.1% w/v aqueous solution), so that the cells were suspended in duplicate aliquots of each detergent. The cells were then incubated at room temperature for 15 minutes.

Labelled carbon dioxide was supplied to the cells as a solution of sodium hydrogen carbonate, in a mixture containing the following:

Tris HCl, 0.1 M, pH 8.0	23 ml
$MgCl_2$, 0.25 M	3 ml
Reduced glutathione	19 mg
$NaHCO_3$	112 mg
Distilled water	8.85 ml
$NaH^{14}CO_3$	10 μCi ^{14}C ml^{-1}

The specific activity of the radioactive mixture was calculated by the addition of 10 μl to each of three scintillation vials already containing 1 ml of absorption reagent (15:35 ethanolamine:2-methoxyethanol, v/v) and immediately adding 10 ml "Optiphase Safe" before scintillation counting.

Aliquots of 0.9 ml of the radioactive mixture were added to both sets of duplicate tubes. This was followed by a further incubation of 10 minutes at 30°C, before initiation of a time course experiment with the addition of 0.3 ml ribulose-1,5-bisphosphate (3 mM) to one of the duplicate tubes. A measure of C_{O2}-fixation by processes independent of ribulose-1,5-bisphosphate was provided by adding 0.3 ml water to the other duplicate tube. Samples were taken from time zero and at 5, 10, 15, 20, 25, 30, 45 and 60 minutes

during a final incubation at 30°C. Samples were immediately placed in a scintillation vial already containing 0.1 ml 6 M phosphoric acid. The acid served to terminate the reaction as well as to enhance the departure of any unfixed carbon dioxide from solution. This process was judged to be complete after the vials had been left overnight at room temperature in a fume cupboard. Residual radioactivity was counted after the addition of "Optiphase Safe".

2.10.4.2 In cell-free extracts.

The assay used essentially the same principle and method as above, except that no detergents were used and the volume of radioactive reaction mixture was increased to 1.8 ml. The volume of cell-free extract used was 200 µl. Specific activities of the reaction mixture and enzyme were calculated as above

2.10.5 Assay of alcohol/aldehyde dehydrogenase.

2.10.5.1 $NAD(P)^+$-linked activity.

The enzymatic oxidation of methanol was measured by the increase in optical density at 340 nm caused by the appearance of NAD(P)H: Due to the broad substrate specificity of the enzyme (Groenneveld *et al.* 1984), it is not possible to differentiate entirely between NAD(P)H appearance due to methanol oxidation, or from the product of that reaction, formaldehyde.

The standard assay mixture contained, in 1000 µl: 20 mM TRIS NaOH pH 10.0, 0.2 µmol $NAD(P)^+$ and 0.1-1.0 mg protein

extract. The cuvette was allowed to equilibrate for 2 minutes. Any endogenous oxidation was followed for a further two minutes before the initiation of the reaction with 10 µmol methanol. Rates of reaction were calculated using a value for the molar extinction co-efficient of NADH of $6200^{-1}cm^{-1}$. Methanol concentration and protein concentration were varied to demonstrate substrate and extract dependence

2.10.5.2 Phenazine methosulphate (PMS) linked activity (pH 9.0, in TRIS buffer).

The enzymatic oxidation of methanol was measured by the increase in optical density at 600 nm caused by the reduction of dichlorophenolindophenol (DCPIP) by reduced PMS. Due to the broad substrate specificity of the enzyme (Groenneveld *et al.* 1984), it is not possible to differentiate entirely between methanol oxidation, or from the product of that reaction, formaldehyde.

The standard assay mixture contained, in 1000 µl: 20 mM TRIS NaOH pH 9.0 (sparged for 1 minute with oxygen-free nitrogen), 0.11 µmol PMS, 0.13 µmol DCPIP, 45 µmol NH_4Cl and 0.1-1.0 mg protein extract. The cuvette was allowed to equilibrate for 2 minutes. Any endogenous oxidation was followed for a further two minutes before the initiation of the reaction with 10 µmol methanol. Rates of reaction were calculated using a value for the molar extinction co-efficient of DCPIP of 22000 $^{-1}cm^{-1}$. Methanol concentration and protein concentration were varied to demonstrate substrate and extract dependence

2.10.5.3 Phenazine methosulphate (PMS) linked activity (pH 9.0, in sodium tetraborate buffer).

The enzymatic oxidation of methanol was measured by the increase in optical density at 600 nm caused by the reduction of dichlorophenolindophenol (DCPIP) by reduced PMS. Cyanide was used to block the catalysis of the reoxidation of reduced electron acceptor by oxygen. Due to the broad substrate specificity of the enzyme (Groenneveld et al. 1984), it is not possible to differentiate entirely between methanol oxidation, or from the product of that reaction, formaldehyde. A second experiment, at pH 7.0, was used to attempt to examine the contribution of aldehyde to the reaction. Ethanol was tried as an alternative substrate in the standard assay.

The assay followed the method developed by Frank and Duine (1990). The standard assay mixture contained, in 1000 µl: 550 µl sodium tertraborate (100 mM, pH 9.0, containing 2 mM methanol), 100 µl DCPIP (0.5 mM), 100 µl KCN (10 mM, in buffer, pH 9.0), 100 µl NH_4Cl (500 mM, in buffer, pH 9) and 50 µl of suitably diluted extract. Additions were made in the indicated order. The cuvette was allowed to equilibrate for 2 minutes. Any endogenous oxidation was followed for a further two minutes before the initiation of the reaction with 100 µl of PMS (10 mM). Rates of reaction were calculated using a value for the molar extinction coefficient of DCPIP of 22000 $^{-1}cm^{-1}$. Methanol concentration and protein concentration were varied to demonstrate substrate and extract dependence. Kinetic data were

calculated with and without cyanide.

2.10.6 Assay of formate dehydrogenase

The enzymatic formation of carbon dioxide and water from formate was measured by the increase in optical density at 340 nm caused by the appearance of NADH:

Formic acid + NAD^+ <=> carbon dioxide + NADH + H^+

The assay followed the method developed by Jollie and Lipscomb (1990). The standard assay mixture contained 880 µl buffer (50 mM MOPS, pH 6.5), 50 µl formic acid (0.1 M in buffer, adjusted to pH 6.5 with 2 M KOH), 10µl NAD^+ (50 mM in buffer) and 25 µl of a suitably diluted aliquot of the cell-free extract. The cuvette was allowed to equilibrate for 2 minutes. The reaction was initiated by the addition of formate after the endogenous rate of the NAD^+ reduction had been measured. Rates of reaction were calculated using a value for the molar extinction co-efficient of NADH of $6200^{-1}cm^{-1}$. Formate concentration and protein concentration were varied to demonstrate substrate and extract dependence and to enable the calculation of a Michaelis constant for the enzyme.

2.10.7 Western blotting of polyacrylamide gels to detect the presence of RUBISCO

Duplicate polyacrylamide gels were prepared with extracts of formate- and MSA-grown cells, occupying 2 tracks each of the gel, and autotrophically-grown *Thiobacillus ferrooxidans* in another two tracks, as a control. Molecular weight markers

were also run in separate tracks. The conditions used are detailed in section 2.9.8. One gel was Coomasie-stained to check that the proteins had separated adequately.

The remaining gel was soaked in transfer buffer (20 mM Tris HCl (pH 8.3), 150 mM glycine, 20% methanol) for 10 minutes and then packed into an electrophoresis electroblotting system (Gradiophore, USA). This enabled the proteins to be blotted onto a Lybond-C nitrocellulose filter (Amersham International plc, Amersham, Bucks) by means of the application of 50 mA for 3 hours.

The nitrocellulose filter was washed in distilled water to remove excess salt and stained with Ponceau S (0.5% (w/v) in 5% (w/v) trichloroacetic acid) for 10 minutes. Several washes with distilled water revealed pink-stained protein bands, allowing the molecular weight markers to be highlighted on the filter with a soft pencil.

All traces of the Ponceau S were removed by repeated washing with 10 ml aliquots of TBS solution (50 mM Tris HCl (pH 8.0), 150 mM sodium chloride). The filter was shaken slowly for 2 hours at room temperature with 20 ml TBS containing 2% (w/v) Marvel milk poowder. The blacking agent was poured off and replaced with 20 ml TBS Marvel containing 4 µl of rabbit anti-wheat RUBISCO antibody, prepared by Dr S. van der Vies, University of Warwick. The filter and antibody were left shaking gently overnight at room temperature.

Unbound antibody was removed from the filter by three washes

with 20 ml TBS containing 0.1% (v/v) Tween 20. A secondary antibody (60 µl goat anti-rabbit peroxidase conjugant IgG in 20 ml TBS Tween 20) was then shaken gently with the with filter for two hours at room temperature. Unbound secondary antibody was removed by two washes with 20 ml TBS Tween 20, followed by two of 50 ml TBS.

The bound primary antibody was revealed by allowing the secondary antibody to catalyse a peroxide/chloronaphthol reaction. The filter was stained with a solution containing 1.5% (w/v) sodium chloride, 1% (v/v) 1 M Tris-HCl (pH 7.5), 0.03% (w/v) chlorohaphthol, 10% (v/v) methanol and 0.05% (v/v) 20 vol. hydrogen peroxide. Black bands appeared on the filter with in one hour, and their position was compared with the molecular weight markers and with the Coomasie-stained dupliacate gel.

2.11 Chemical syntheses

2.11.1 Derivitisation of methane sulphonate.

Methane sulphonic acid has a boiling point of more than 300 °C, and so is unsuitable for conventional gas chromatographic detection techniques. However, it is sometimes possible to prepare a derivative of sulphonates which have a boiling point below 200 °C (Eagles & Knowles 1971). If this preparation yields a reproducible quantity of product proportional to the reactant, this may form the basis of an assay. Two conventional techniques were assessed: silylation and acylation.

2.11.1.1 Trimethyl-silyl Derivatives of Methane Sulphonic Acid.

Trimethylsilylimadizole (TMSI) reacts with sulphonates to form a silyl ester, (see fig 2.11.1.1). To effect derivitisation, the method used by Eagles and Knowles (1971) was used. A small quantity (400 mg) of the sodium salt of methane sulphonic acid was placed in an autosampler vial (Waters Ltd) and incubated overnight at room temperature with 300 µl 1-(trimethylsilyl)imidazole. Volatile products of the reaction were assayed using gas chromatography.

2.11.1.2 Acyl derivatives of methane sulphonate

The derivatives were prepared according to the method of Bakke *et al.* (1989). Methane sulphonic acid was dissolved in MinE at concentrations varying between 1 and 30 mM. These solutions were applied to a column of anion exchange resin (AG-1 X8, chloride form, Biorad, USA) and eluted sequentially with 0.1 and 0.5 M HCl. The MSA coeluted with the 0.5 M HCl and these eluates were collected and dried in 2 ml autosampler vials (Waters Ltd). The vials were sealed with Teflon lined rubber septa. The acylchloride of MSA was formed by the addition of an equal volume of thionyl chloride, followed by incubation overnight at 95 °C. Excess thionyl chloride was removed in a fume hood by allowing nitrogen to bubble through the samples for 10-15 minutes. The addition of an equal volume of aniline formed the *N*-(phenyl)-methanesulphonamide. This was extracted into ether (10 ml) after acidification with 1 M HCl. Concentrations of

N-(phenyl)-methanesulphonamide in the samples were assayed by gas chromatography.

2.11.2 Preparation of methanol-free formaldehyde

Formaldehyde solutions supplied by chemical companies necessarily contain methanol as a stabilising agent. A solution containing formaldehyde alone can be prepared in the following manner.

Narrow glass tubes of low melting point were filled with 1.2 g paraformaldehyde and made up to 3 ml with distiled water. The open end of the tube was sealed over a Bunsen burner to form an ampoule. The ampoule was shaken thoroughly to make an even suspension of the paraformaldehyde. The paraformaldehyde was depolymerised to a 40% (w/v) formaldehyde solution by autoclaving overnight at 15 psi and 126 °C.

2.11.3 Preparation of trimethylsulphonium chloride (TMS-Cl).

The method followed that of Wagner *et al.* (1972). A 5 M aqueous solution of trimethylsulphonium iodide was passed through a column containing Dowex 1-chloride anion exchange resin, and the chloride solution collected. The concentration of TMS-Cl was checked by total organic carbon analysis.

2.12 Special Chemicals and Radiochemicals

All chemicals, with the exception of those below, were supplied by Aldrich, Sigma or BDH and were of ANALAR grade

(or equivalent) or above.

Radiochemicals were all supplied by Amersham International (Amersham, England) with the exceptions of ^{14}C-labelled methane sulphonate, which was kindly supplied by Dr Bakke at the University of California.

CHAPTER 3:

ENRICHMENT AND ISOLATION OF CARBON DISULPHIDE USERS

3.1 Introduction

Enrichment of environmental samples for bacteria capable of degrading CS_2 and using it as sole carbon and energy source has been attempted (Smith and Kelly 1988b), but without success. Those bacteria capable of growing on CS_2 (Smith and Kelly, 1988b; Smith and Kelly, 1990), oxidising it (Butler *et al.*, 1969) or using it as a sulphur source (Rajagopal and Daniels, 1986) were not isolated with in the laboratory from CS_2 enrichments. It is clear from the molecular structure and thermodynamic properties of CS_2 that it can only be used as a chemolithotrophic energy source for autotrophic growth. This coupled with the compound's toxicity could restrict the possible isolates to a few specialised organisms such as *Thiobacillus* or *Hyphomicrobium*. Thus even though CS_2 could be considered a compound ubiquitous in nature, it was decided to attempt isolations from soil that had been repeatedly contaminated with CS_2 over a number of years rather than from previously unexposed soil or water.

Soil samples were collected, during the winter of 1989/1990 from various points around the Courtaulds Sulphur Chemicals plant at Stretford in Manchester (see figure 3.1 for exact location). Sampling during the summer would have been closer to an ideal, the warmer weather perhaps increasing biological activity, but special permission had to be obtained to gain access to the site and December was the only time convenient to Courtaulds plc.

Stretford was chosen as it had had a long history of CS_2 contamination. The areas sampled were characterised by the total absence of plant and animal life. An indication of the levels of

CS_2 can be gained from the problems encountered during construction work, carried out just south of the sampling site. It was necessary to remove some of the soil by mechanical digger. However, as the metal bucket entered the earth, sparks from stones ignited the CS_2 contaminating the soil, causing an explosion of sufficient force to blow the bucket out of the hole it had created.

There are several means by which CS_2 could enter the pedosphere. CS_2 is manufactured and stored at the plant, so local atmospheric levels of CS_2 are higher than normal (see table 1.2.1), and this would be reflected in larger quantities of CS_2 in rainfall - CS_2 vapour is heavier than air and probably would have accumulated locally. Another means of entry of the sulphide to the soil was by spillage. As tanker lorries carrying CS_2 are loaded or unloaded, they are parked in a pond, and this pond water is allowed to run off onto nearby waste ground. The acceptable level of spillage is not recorded (Courtaulds plc 1984), but an apocryphal estimate puts the figure at around 5% of the load. Lastly, "bund", a gravelled absorbent compound, from inside and underneath the CS_2 reaction tanks is disposed of on the same patch of waste ground. Since the whole site is used in the manufacture of sulphur chemicals in general, it is likely that this would cause a selective pressure in favour of those organisms capable of metabolizing or tolerating organosulphides.

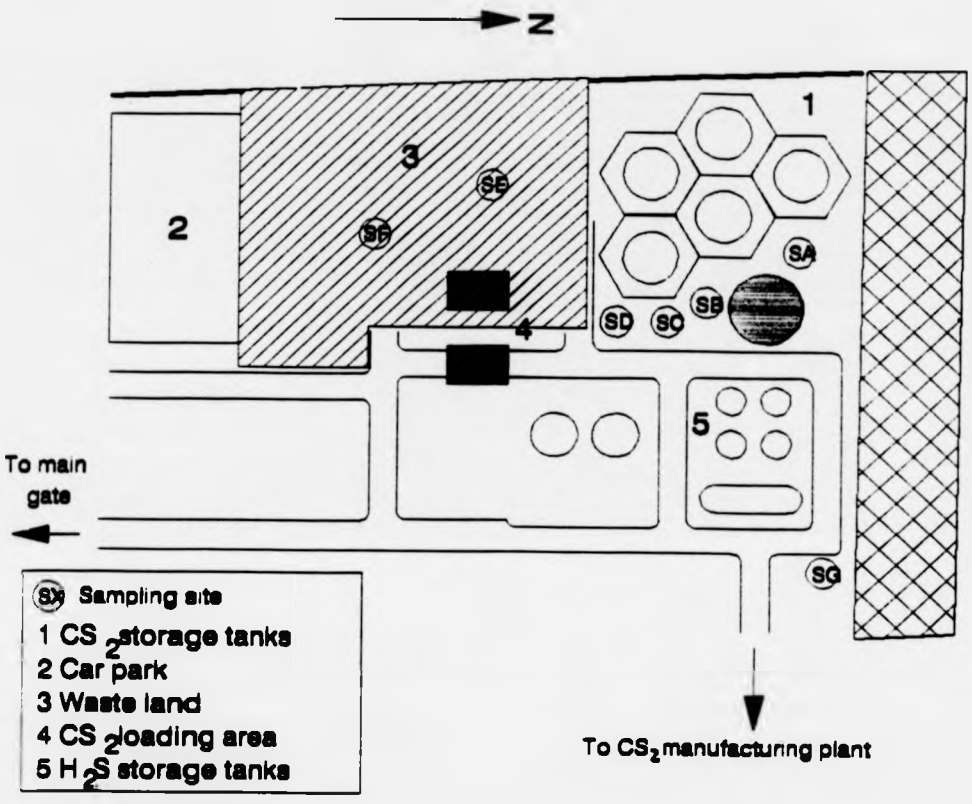


Figure 3.1 Soil and surface water sampling sites at Courtaulds Sulphur Chemical Ltd., Stretford, Manchester.

Once soil, containing organisms that had been regularly exposed to CS_2, had been obtained, enrichment was continued in liquid culture.

3.2 Enrichment culture

Seven enrichment cultures were initiated with inocula from the following sources:

SA, soil from beside CS_2 storage tanks;
SB, soil from beside storage tanks;
SC, water from small pool beside storage tanks;
SD, soil from beside storage tanks;
SE, run off from tanker loading pools;
SF, discarded bund;
SG, soil from canal-side grassy bank.

Approximately 10 ml was added to each flask. The soils themselves were very different in appearance. SA, SB and SD were dark, granular soils, whereas SF was beige and clay-like, due to the regular heavy input of bund. The surface waters collected were turbid, even after being allowed to settle overnight.

Since toxicity from CS_2 was likely to be a problem during enrichment, it was necessary to monitor bacterial populations in the flasks and so ensure that the sulphide did not sterilise the flasks. Samples were taken each week from the enrichment cultures and streaked out onto nutrient agar (NA), T. versutus medium and, later in the experiment, a more acidic version of the T. versutus medium. The pH of the media was also monitored throughout the experiment. It was measured weekly using a pH probe, but day-to-day pH adjustments were made according to the colour of an indicator in the medium, bromothymol blue. In this way the pH of the media was returned to pH 7 at regular intervals.

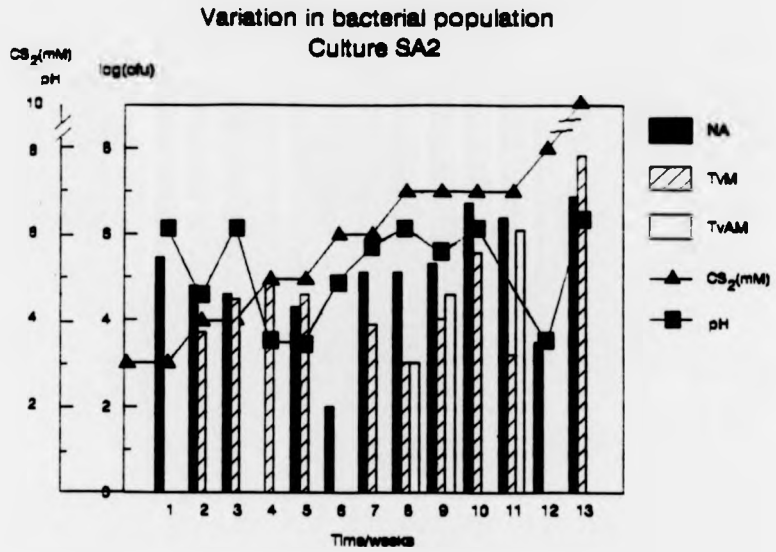
The possible toxic effects of CS_2 were also taken into consideration when adding the compound as a substrate. Carbon disulphide was added to the enrichments weekly, and every two weeks the amount added was increased by 1 mM.

3.3 Variation in bacterial populations in isolation media

After 25 weeks incubation, the cultures contained a varied population of bacteria. This included thiobacilli (growing on thiosulphate medium at pH 4.5 and 7.0) and several heterotrophs, which included a colony form consistent with a nocardiaform actinomycete. All the cultures could completely metabolise 10 mM CS_2 from the medium within one week (see Chapter 4.2.2).

The proportions of the heterotrophic population to the thiobacilli varied with time (see figures 3.3a, b, c, d).

The factors that appeared to have affected bacterial populations were similar in all flasks, but are best illustrated by flask SA. This was split into two enrichment experiments (the flasks renamed SA1 and SA2) after a glassware accident. SA1 contained media retrieved from the broken flask, SA2 contained that retrieved from the bottom of the incubator.



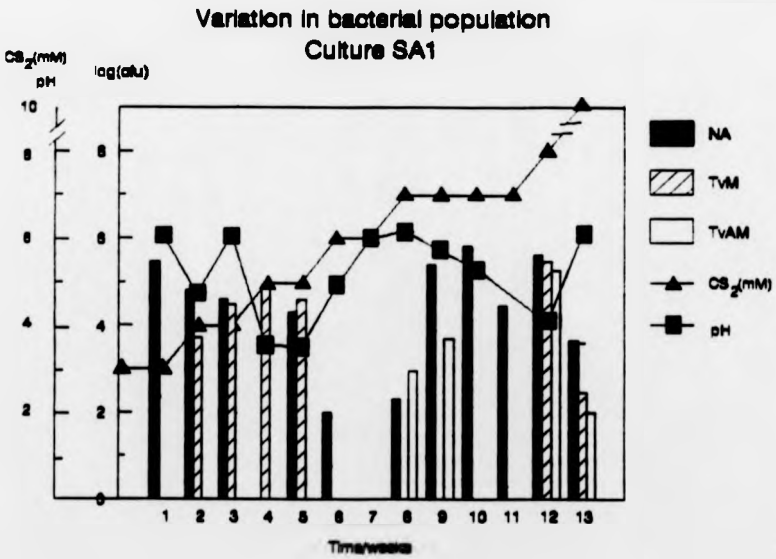
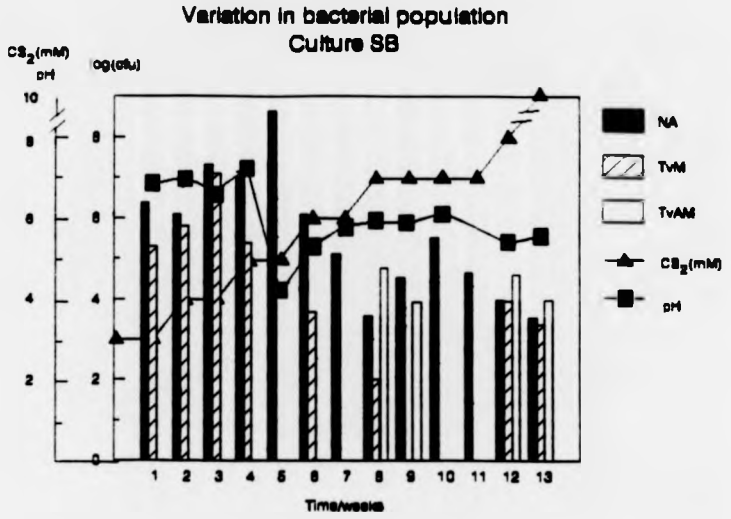


Figure 3.3a Variation in numbers of heterotrophs and "thiobacilli" in CS_2 enrichment cultures SA1 and SA2. Lower detection limit of bacteria: 10 ml $^{-1}$. NA = nutrient agar, TvM = Thiobacillus versutus medium. TvAM = acidic T. versutus medium.



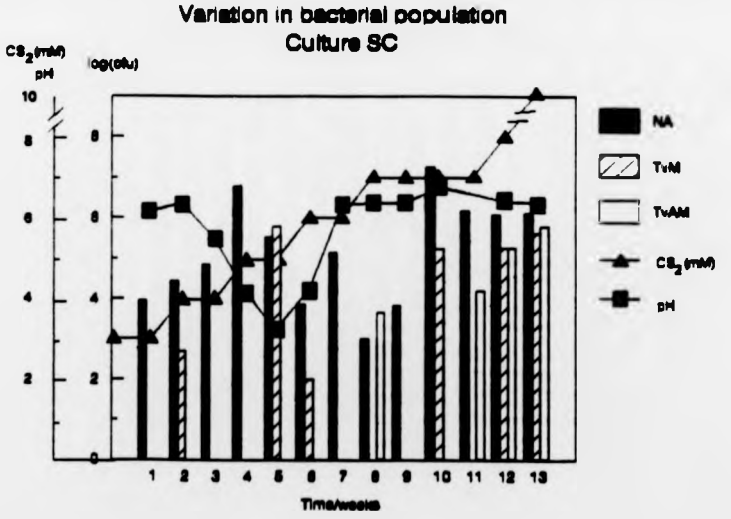


Figure 3.3b Variation in numbers of heterotrophs and "thiobacilli" in CS_2 enrichment cultures SB and SC. Lower detection limit of bacteria: 10 ml $^{-1}$. NA = nutrient agar, TvM = Thiobacillus versutus medium. TvAM = acidic T. versutus medium.

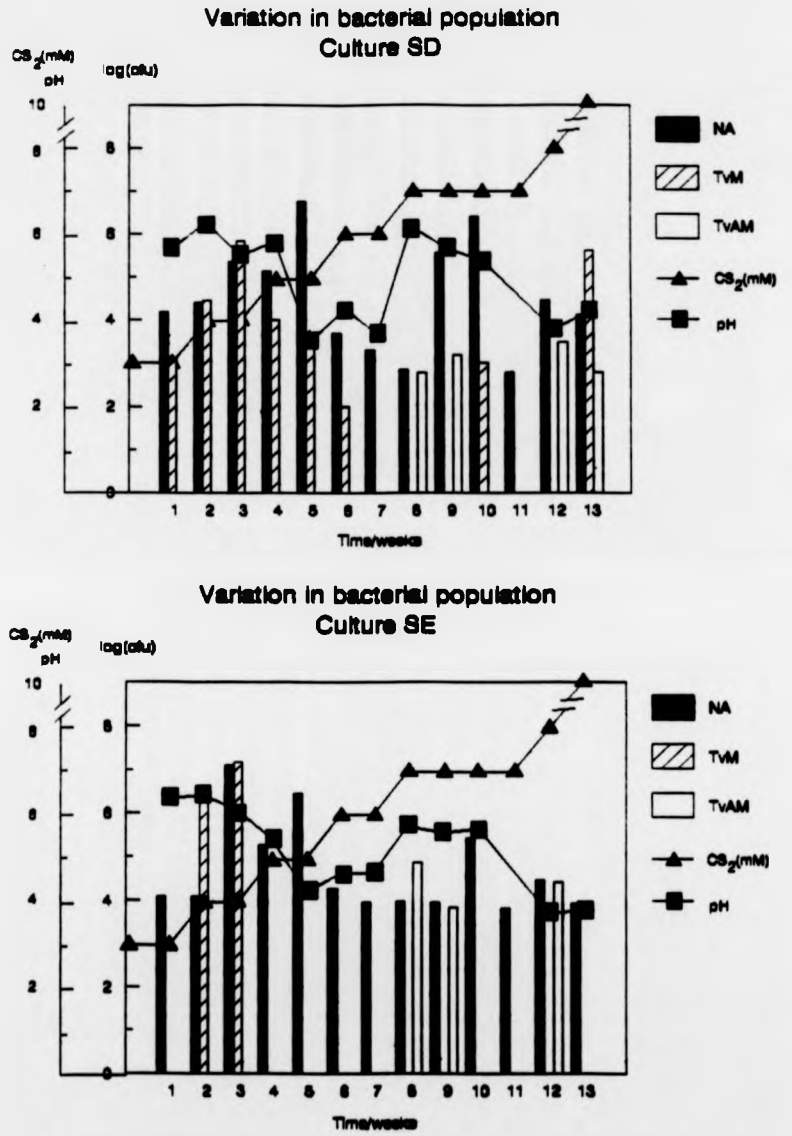


Figure 3.3c Variation in numbers of heterotrophs and "thiobacilli" in CS_2 enrichment cultures SD and SE. Lower detection limit of bacteria: 10 ml $^{-1}$. NA = nutrient agar, TvM = Thiobacillus versutus medium. TvAM = acidic T. versutus medium.

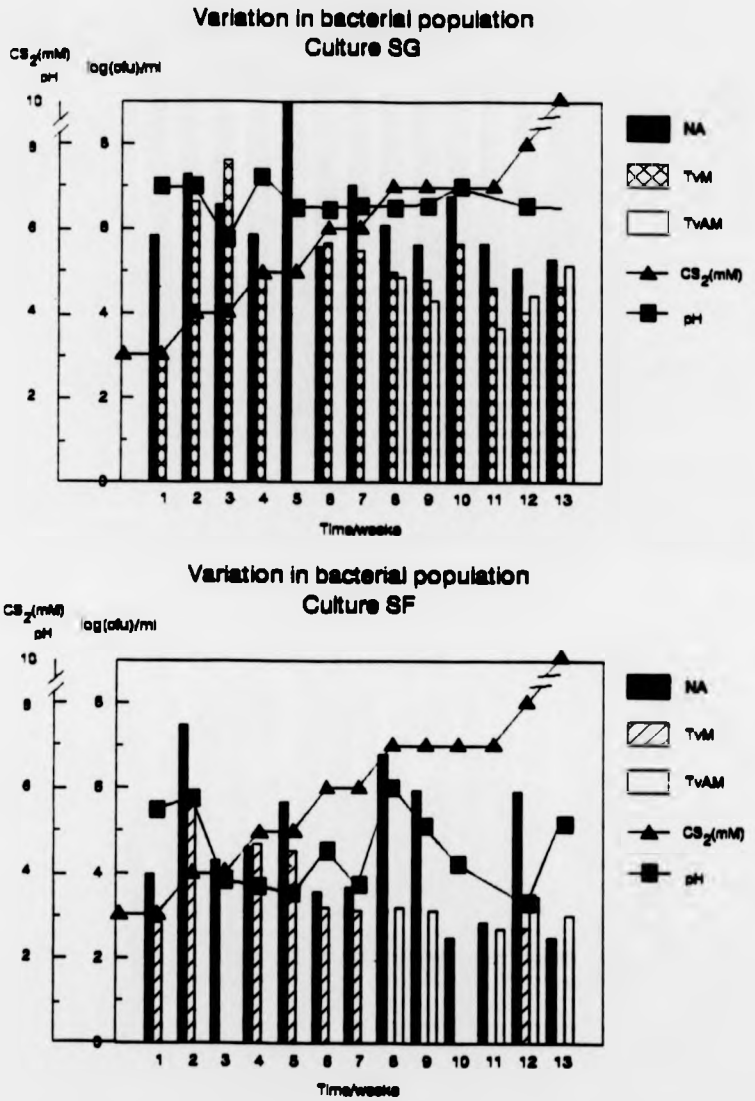


Figure 3.3d Variation in numbers of heterotrophs and "thiobacilli" in CS_2 enrichment cultures SF and SG. Lower detection limit of bacteria: 10 ml^{-1}. NA = nutrient agar, TvM = Thiobacillus versutus medium. TvAM = acidic T. versutus medium.

The heterotrophic population (as detected on nutrient agar) was the most susceptible to pH. In general, a fall in pH was mirrored by a fall in the numbers of heterotrophs. Conversely, the numbers of thiobacilli (as detected on TvM and TvAM) increased as the pH declined.

3.4 Isolation of CS_2-users

A primary objective in the enrichment process was to select for an organism with better growth characteristics on CS_2 then *Thiobacillus* TK-m. Despite screening 250 colonies from solid media, 42 of which appeared to use CS_2 while growing on liquid media, this objective was not fulfilled. The mixed culture SE used CS_2 faster than previously reported for a bacterial culture (Smith and Kelly, 1988b and Chapter 4), but pure cultures could not be obtained. The failure to isolate pure cultures could probably be attributed to the properties of CS_2 itself, particularly its toxicity. Screening a large number of colonies (each presumably with their own growth criteria), yet supplying them with enough CS_2 for energy, balanced against restricting the CS_2 in case of toxicity problems is a difficult task, made harder by the isolation procedures that must be used because of the CS_2.

CHAPTER 4:
BACTERIAL METABOLISM OF CARBON DISULPHIDE

4.1 Introduction

The possible primary metabolites of CS_2, such as COS and hydrogen sulphide (H_2S), are characterised by their volatility. Thus it is often possible to detect intermediates in the headspace above a culture and to quantify them by gas chromatography. If the detector used is selective for sulphur compounds, as is the case with the flame photometric detector (FPD), then volatile sulphur compounds can be detected at concentrations equivalent to parts per billion.

4.2 The metabolism of carbon disulphide by mixed cultures

4.2.1 Growth of the Harfoot culture MVA1 on CS_2

The Harfoot cultures are largely heterotrophic (see chapter 5), and growth has been reported on carbon disulphide as sole source of carbon and energy. However, when diethyl sulphide-grown organisms are inoculated into cultures containing 1 or 2 mM CS_2, although growth was detected (presumably due to the CS_2), the results are not consistent (see figure 4.2.1).

The 50ml cultures were grown at 30 °C without shaking, and incomplete mixing may have accounted for the difference between duplicates. However, these were the only results obtained showing growth. Three similar experiments failed altogether. A more likely explanation for the discrepancy is that the heterotroph does not grow on CS_2, and that a minor member of a mixed population within MVA1 partially

metabolizes the CS_2, and the heterotrophs survive on excreted products (More evidence for this view is presented in chapter 5). Thus the growth of the heterotrophs is dependent on the presence of another organism. In turn the proportion of this CS_2 user, and thus the effective inoculum size, may vary with unknown factors.

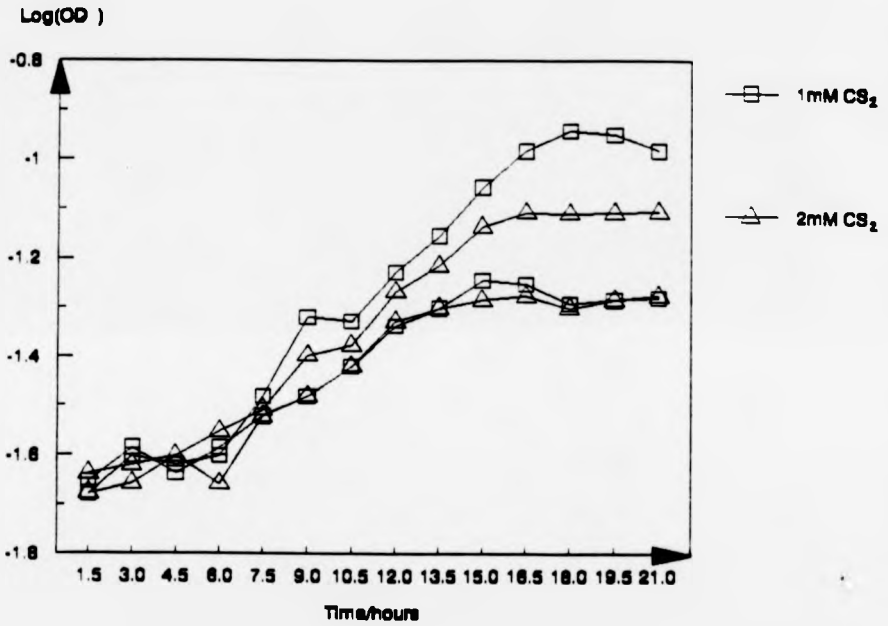


Figure 4.2.1. The growth of the Harfoot culture MVA on 1 and 2 mM CS2 (duplicate cultures). "□" = OD_{440} of MVA grown on 1 mM CS_2. "△" = OD_{440} of MVA grown on 2 mM CS_2.

4.2.2 The metabolism of CS_2 by cultures SA1-SG

4.2.2.1 Rates of use of CS_2 by impure cultures

The rate of use of CS_2 by the cultures was determined by a 175 hour time course experiment, with 10 µl headspace gas from the cultures injected into the GC/FPD. The experiment

was initiated with the injection of 4 mM CS_2, which in some cases supplemented CS_2 that had not been metabolized (see table 4.2.2.1). Thus initial CS_2 concentrations were between 3.92 mM and 6.27 mM. In all cases residual COS/H_2S was also present.

	Culture							
	SA1	SA2	SB	SC	SD	SE	SF	SG
Time	0.68	1.07	3.73	4.73	5.47	5.73	5.93	6.17
CS_2 (mM)	6.05	5.90	5.63	6.27	5.87	3.92	5.67	5.81
H_2S/COS	12.46	14.92	14.27	6.55	12.90	15.31	6.959	14.25
Time	20.92	21.57	22.00	22.35	22.73	23.02	23.40	23.75
CS_2 (mM)	4.79	4.27	5.04	6.10	5.57	0.91	6.19	5.17
H_2S/COS	14.18	15.41	15.10	8.010	13.42	15.23	7.143	14.92
Time	70.88	71.03	71.25	71.48	71.77	72.05	72.18	72.43
CS_2 (mM)	0.86	2.27	3.66	5.52	4.82	0.48	5.61	3.50
H_2S/COS	15.01	15.22	15.30	8.634	14.37	15.14	7.916	15.29
Time	92.50	92.67	93.52	92.12	93.95	94.25	94.53	94.93
CS_2 (mM)	0.82	1.83	3.04	4.98	3.92	0.35	4.85	1.46
H_2S/COS	14.77	15.00	14.99	8.33	14.72	15.29	7.92	14.95
Time	119.1	119.2	119.4	119.5	119.7	119.9	120.0	120.2
CS_2 (mM)	0.68	1.66	2.59	4.52	3.41	UR	4.43	0.66
H_2S/COS	13.99	14.82	14.79	8.677	14.98	9.322	8.150	14.00
Time	170.3	170.5	170.6	170.8	171.0	171.3	171.5	171.8
CS_2 (mM)	0.48	0.95	2.08	4.02	0.28	UR	3.93	0.38
H_2S/COS	12.56	13.35	14.03	8.707	12.30	12.04	7.734	12.19

Table 4.2.2.1 The use of CS_2 by cultures SA1 to SG. H_2S/COS figures are given as the $\log_e$ of the peak area. UR = under range, i.e. less than 0.2 mM. Under range readings still show an unquantifiable CS_2 concentration.

The concentrations of CS_2 and the size of the peak associated with H_2S and COS were plotted against time. From these graphs (Figures 4.2.2.1 and 4.2.2.2) it was possible to calculate an approximate rate of use of CS_2 by linear regression analysis of the points on the graphs that

appeared to form straight lines. The control of 50 ml medium C with 4 mM CS_2 is omitted in each case for clarity.

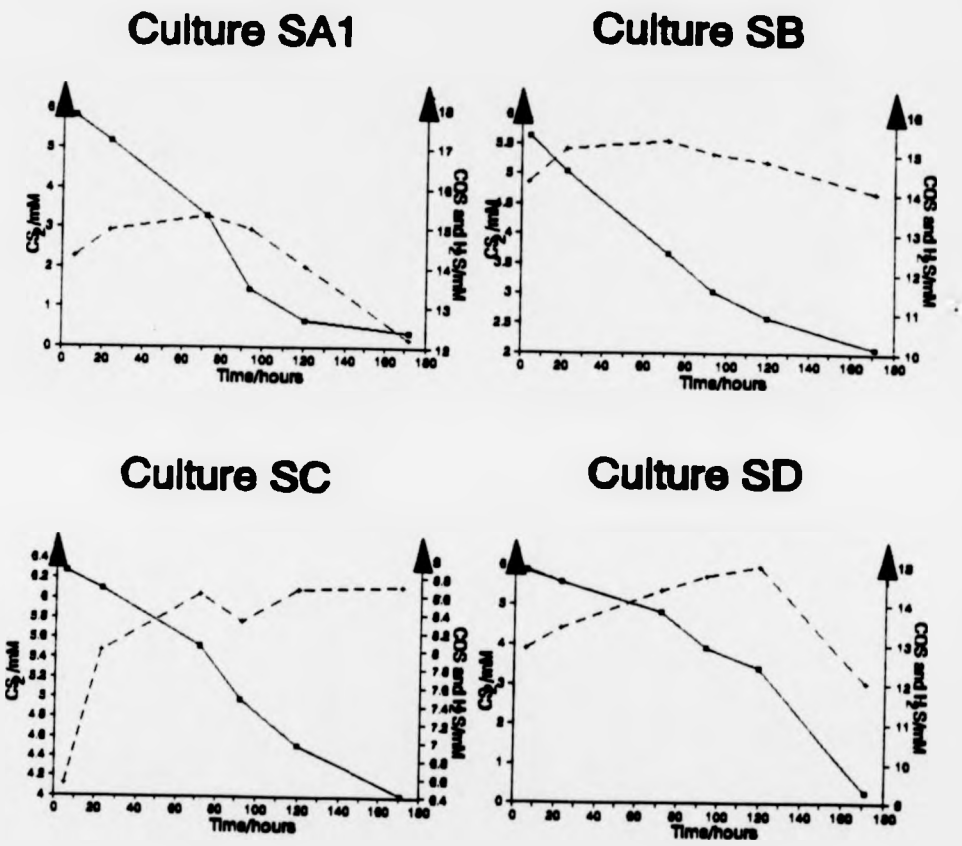


Figure 4.2.2.1 Use of CS_2 by enrichment cultures SA1, SB, SC and SD and the presence of H_2S and/or COS. "+" = concentration of COS/H_2S in mM, "■" = concentration of CS_2 in mM

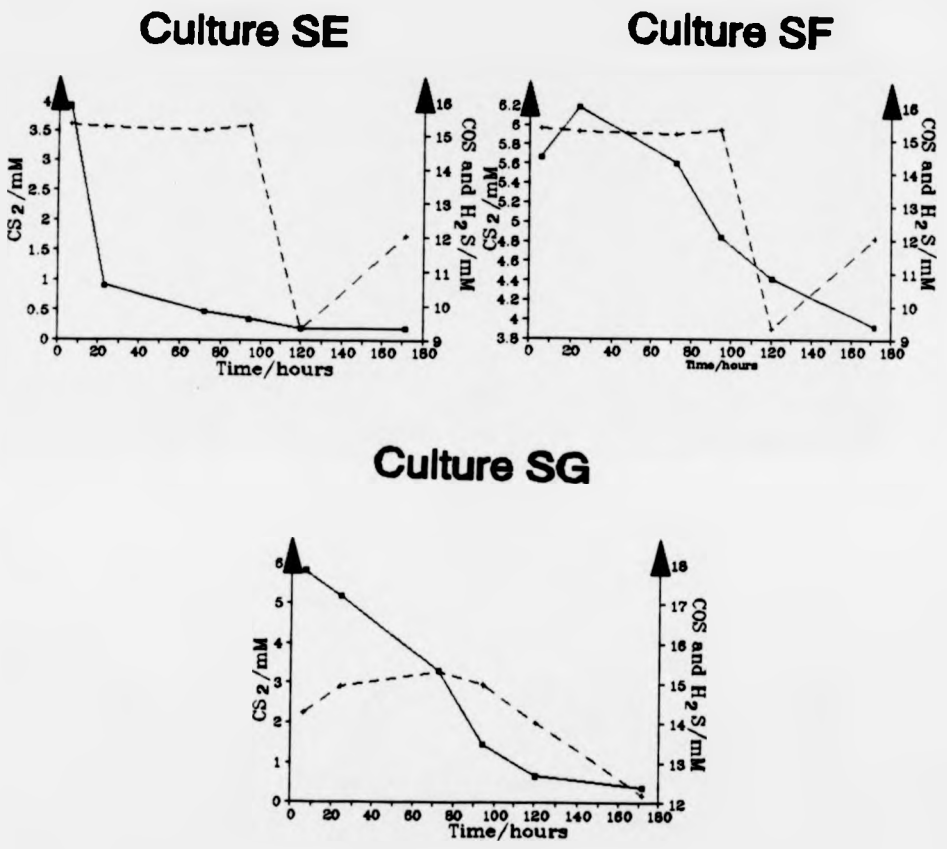


Figure 4.2.2.2 Use of CS_2 by enrichment culture SE, SF and SG and the presence of H_2S and/or COS.

The results of these linear regressions are shown in table 4.2.2.2.

Culture	Rate of CS_2 disappearance (μmol l^{-1} $hour^{-1}$)
SA1	75
SA2	50
SB	27
SC	14
SD	32
SE	170
SF	16
SG	46

Table 4.2.2.2 Rates of use of CS_2 by impure cultures.

Although the curves generated varied from flask to flask, they had common features: as CS_2 concentration decreased, the H_2S/COS peak increased and as the experiment continued, this peak declined as well. This is better illustrated by an experiment using 10 ml of the culture SG inoculated into 40 ml of medium C (see figure 4.2.2.3).

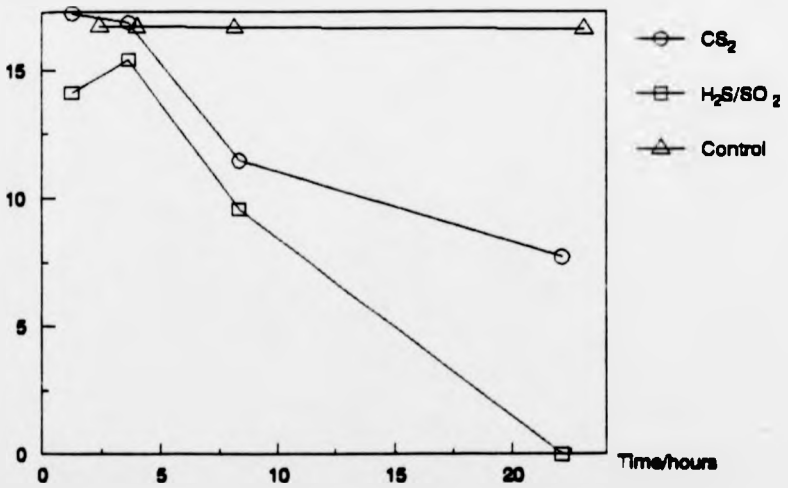


Figure 4.2.2.3 Use of CS_2 by a subculture of the enrichment culture SG and the presence of H_2S and/or COS.

The peak associated with H_2S and COS rose as the CS_2 disappeared (0-4 hours), and then decreased to zero (i.e. not only below the quantifiable limit as for CS_2, but to an undetectable limit). The subculture oxidised CS_2 at a significantly higher rate than the enrichment cultures (445 μmol l^{-1} $hour^{-1}$ as oppossed to 170 μmol l^{-1} $hour^{-1}$ for culture SE). This was probably due to the dilution of an inhibition factor or factors accumulated from the heterologous population (see section 3.2).

4.2.2.2 Detectable products during growth on CS_2

The products found in the enrichment flasks are shown in table 4.2.2.3. None were quantifiable. In one of the flasks (SC) sulphide was in sufficiently high concentration to turn the medium black. H_2S was detected by odour and confirmed by the ability of headspace gas to turn damp lead acetate-saturated filter paper black. Carbonyl sulphide was measured with H_2S in the GC/FPD. Sulphur was visible as yellow granules floating on the surface of the medium, but was not detectable by the

Culture	Detectable products			
	H_2S/COS	Sulphide	Sulphur	Acid
SA1	+	-	+	+
SA2	+	-	+	+
SB	+	+	-	+
SC	+	+	-	+
SD	+	-	-	+
SE	+	-	+	+
SF	+	-	+	+
SG	+	-	-	+

Table 4.2.2.3 Products detectable during the growth of impure cultures on CS_2.

chemical test for sulphur (see chapter 2), possibly because of interference by sulphides or other organosulphur compounds.

The mode of metabolism indicated by these products differs little from previously reported work. Sulphur was noted by Smith and Kelly (1988b) in colonies of TK-m. This strain also produced carbonyl sulphide during batch culture. Hydrogen sulphide was only noted during anaerobic incubation, but slow shaking of 50 ml of medium in a 250 ml Erlenmayer flask may not lead to adequate aeration.

4.3 Discussion

There are particular problems with using headspace GC/FPD to detect carbon disulphide. First, the preparation of standards: the easiest way is to prepare standard solutions, made up in the medium that will be used in the experiment. As long as the volume of air above the liquid in the experiment is the same as the volume above that of the standard, and both flasks are maintained at 30°C, then the effect of a partition coefficient between liquid and gas can be ignored and the concentration of CS_2 in the headspace will be directly proportional to the concentration of CS_2 in the liquid.

However, although this method provides a standard curve for freshly prepared CS_2 solutions, it will give false results in a time course experiment. Carbon disulphide, in common with other organosulphides, is absorbed into rubber, specifically, in this case, the rubber Subaseal used as a

septum and bung in the flask. This means that to account for this absorption, new Subaseals must be used for each standard and experimental flask, to ensure that absorption is equal. Similarly the standard solution must be incubated in the same way as the experimental flask, to ensure the continuation of equal absorption, and resampled every time an experimental reading is taken.

The second problem with using GC/FPD for any organosulphur compound is the detector response. As can be seen from figure 4.3.1 there is no simple relationship between peak area (as calculated by the GC integrator) and CS_2 concentration. Between 1 and 10 mM (Data shown only for 0.2 - 4 mM), the relationship is exponential, so the natural logarithm of the area is directly proportional to CS_2 concentration. However, below 1 mM, the detector response changes to give a sigmoid calibration curve, and so it is best to read straight off the graph, rather than apply a multiplication factor to $\log_e$(area). At concentrations below 0.2 mM, the equipment's sensitivity makes the acquisition of reproducible results very difficult (using the method of preparing standard curves above), so in this work the detection limit of the GC/FPD is taken as 0.2 mM. However, it should be noted that non-quantitative results can be obtained at sulphide concentrations much less than 0.2 mM.

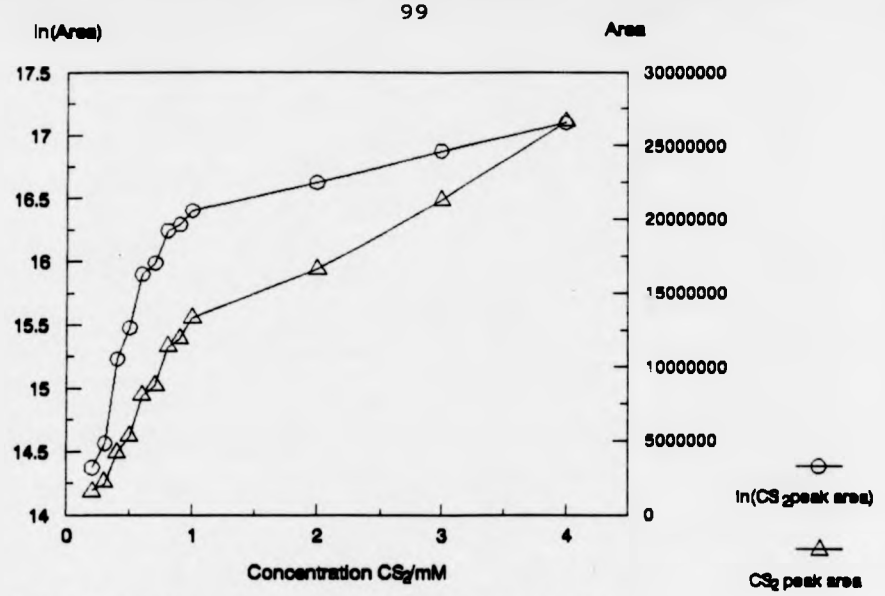


Figure 4.3.1 Detector reesponse to varying concentrations of CS_2.

The last problem concerns the particular column used in separating the organosulphur compounds. It was chosen to have a good response to all those compounds used throughout the work, such as DMS, DES and CS_2. Separation in time between peaks increases with temperature but even if the column was run at its maximum working temperature (150°C), COS and H_2S appear as one peak. It was impossible to make any quantitative judgement from the COS/H_2S peak, as the detector response for each compound was different:

Compound	Concentration	Peak Area
COS	1 mM	1625090
H_2S	1 mM	2492045

Table 4.3 GC/FPD response to two organosulphur compounds.
Figures are taken from the average of three headspace GC/FPD results. Solutions were prepared as described in the methods section.

Thus an experimental peak could be made up of COS and H_2S in

any proportion. Furthermore, the peak size will not be related to the concentration of sulphide in the form of either H_2S or COS.

Taking the above limitations into account, the results presented below have, in some cases, a large margin of error. This applies particularly to the use of CS_2 by the cultures SA-SG. Since the flasks were employed continuously for eight months, then obviously the Subaseals will have radically different organosulphur absorbing properties than the standards used against them.

Notwithstanding the above comments, the products identified by GC/FPD were definitely present in the flasks, if only in non-quantifiable form. Since they do not differ radically from intermediates detected by other workers (Chengelis and Neal 1986; Smith and Kelly 1988b), it may be reasonable to assume that the organisms performing primary degradation of the CS_2 used enzymatic steps similar to that of *Thiobacillus thioparus* TK-m. This is supported by the presence of organisms in the medium that grow on thiosulphate media (TvM and TvAM).

CHAPTER 5:
CHARACTERISATION OF THE HARFOOT CULTURE COLLECTION

5.1 Introduction

The bacteria isolated by Dr C. Harfoot (University of Waikato) from Antarctic and New Zealand locations were selected on their ability to grow on diethyl sulphide (DES), dimethyl sulphide, dimethyl disulphide or thiosulphate. The cultures were unusual in that no organism has previously been isolated with the ability to grow on DES and in most cases the cultures possessed morphologies not previously associated with the ability to use sulphur compounds (see Chapter 2, table 2.1.2). The majority were Gram positive coryneform bacteria. It was hoped that an examination of the isolates would reveal whether DMS and DES were metabolized using the same enzymes and if this mechanism was the same as that found in organisms such as *Thiobacillus thioparus* strain E6 (Smith and Kelly 1988a).

5.2 Characteristics of the Harfoot culture collection

Before this work started, the best characterized strain was the Gram positive rod, MVA. It was known to grow on DMS (on which it was isolated), DES, CS_2 acetate and nutrient agar. Diethyl sulphide-grown cells could oxidise DMS, DES, dipropyl sulphide, DMDS, DEDS, ethane thiol, butane thiol, CS_2, sodium sulphide, formaldehyde and ethanal (C. Harfoot and D.P.Kelly, unpublished results). The data on the growth range of the bacterium, and others in the collection, were increased by the use of the API20B system (Table 5.2.1a and 5.2.1b). This also had the benefit of showing that the cultures of similar morphology were not the same strain.

This was particularly important with regard to MVA. Close examination of agar plates containing acetate revealed two colony forms, one more mucoid than the other, and these cultures were renamed MVAm (mucoid) and MVAe (entire). Cultures of these colony forms were maintainable on both acetate and DES. Both colony forms were present in subcultures of the original slopes.

Culture	MVAm	MVAe	MVA/2	EV1	E1/3	MWD	M4/20	M1/5S
Growth on:								
DES	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+
Nutrient agar	+	+	+	+	+	+	+	+
Acid from:								
Saccharose	-	-	-	+	-	-	-	+
L (+) Arabinose	-	-	-	+	+	-	-	-
Mannitol	+	+	+	+	-	+	-	+
Fructose	+	+	+	+	+	+	+	+
Glucose	+	-	-	+	+	+	-	+
Maltose	-	-	-	+	+	-	-	-
Starch	-	-	-	+	-	-	-	-
Rhamnose	-	-	-	+	-	-	-	-
Galactose	-	-	-	+	+	-	-	-
Mannose	-	-	-	+	+	-	-	-
Sorbitol	+	+	-	+	-	-	-	+
Glycerol	+	+	-	+	-	-	+	+
Alkali from:								
Urea	+	+	+	-	+	-	+	+
Citrate	-	-	+	+	+	+	+	-
Gelatine hydrolysis	-	-	-	+	+	-	-	-
Nitrate reductase	-	-	-	-	-	+	-	-
ß galactosidase	-	-	-	+	-	-	-	-
Indole	-	-	-	-	-	+	-	-
Hydrogen sulphide	-	-	+	-	+	-	-	-
Acetoin	-	-	-	-	+	-	-	-
Oxidase	-	-	+	-	+	+	-	+
Catalase	-	-	-	-	-	-	-	+
Mobility	+	+	+	-	+	-	+	+
Gram stain	+	+	+	+	+	+	+	+
Coccoid?	-	-	-	-	-	-	-	-
Sporulation	-	-	-	-	-	-	-	-

Table 5.2.1a Characteristics of dominantly Gram positive coryneform Harfoot cultures.

Culture	TF1	TF2	D4
Growth on:			
DES	+	+	+
Acetate	+	+	+
Nutrient agar	+	+	+
Acid from:			
Saccharose	-	+	-
L (+) Arabinose	-	+	-
Mannitol	+	+	-
Fructose	+	+	+
Glucose	-	+	-
Maltose	-	+	-
Starch	-	+	-
Rhamnose	-	+	-
Galactose	-	+	-
Mannose	-	+	-
Sorbitol	+	+	-
Glycerol	+	+	-
Alkali from:			
Urea	+	-	+
Citrate	-	-	+
Gelatine hydrolysis	-	-	-
Nitrate reductase	-	-	-
ß Galactosidase	+	+	-
Indole	-	-	-
Hydrogen sulphide	-	-	-
Acetoin	-	-	-
Oxidase	-	-	-
Catalase	nt	nt	nt
Mobility	nt	nt	nt
Gram stain	-	-	-
Coccoid?	-	-	-
Sporulation	-	-	-

Table 5.2.1b Characteristics of selected Gram negative Harfoot cultures.

The growth of MVA and other cultures was enhanced by allowing the organisms to grow in a stationary liquid culture rather than with shaking. This is shown in figure 5.2.1 and discussed further below.

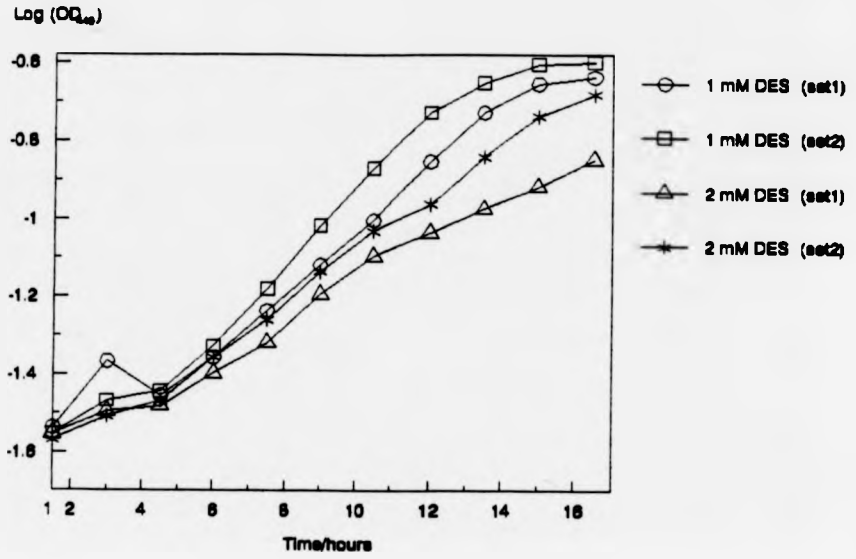


Figure 5.2.1 The growth of MVA on 1 and 2 mM DES. Set 1 (○ and △) were flasks containing 50 ml MinC grown at 30 °C with shaking at 150 rpm, set 2 (□ and ✳) were incubated without shaking. "○" = set 1, 1 mM DES, "□" = set 2, 1 mM DES, "△" = set 1, 2 mM DES and "✳" = set 2, 2 mM DES.

5.3 Classification of the Harfoot cultures

The coryneform members of the Harfoot culture collection were classified with the aid of 23 biochemical tests in a method devised by Sieler *et al.* (1980). These 23 tests were selected by these workers from 135 characteristics used in the identification of coryneforms from activated sludge. They form a numerical taxonomy allowing the clustering of

coryneforms into 5 groups, each with subgroups (see table 5.3.1). Similar tests were carried out on the Gram positive members of the Harfoot culture collection. The agar that Sieler *et al.* (1980) used (Ionagar) was unavailable, and so Difco Bacto agar was substituted.

Group	A	A1	A2	B	B1	B2	C	C1	C2	C3	D	E	E1	E2
Similarity (%)	84	88	88	84	88	88	84	88	88	88	84	84	88	88
Utilization of:														
Pyruvate	+	+	(+)	d	d	d	(+)	+	+	d	d	+	+	+
DL-lactate	+	+	(+)	-	(-)	-	(+)	+	+	d	-	+	+	+
Propionate	+	+	(+)	-	(-)	-	+	+	+	+	+	+	+	+
2-ketogluconate	(-)	-	-	-	-	-	-	-	-	-	-	d	-	+
5-amino-valerate	+	+	(+)	-	-	-	d	d	d	-	-	(-)	+	-
DL-malate	+	+	+	-	(-)	-	(-)	d	d	(-)	-	+	+	+
Succinate	+	+	+	-	(-)	-	d	d	+	d	-	+	+	+
Citrate	+	+	(+)	-	-	-	+	+	(+)	(+)	(-)	+	+	+
4-hydroxybenzoate	+	+	(+)	-	-	-	-	-	-	-	-	+	+	+
L-aspartate	+	+	+	-	(-)	-	d	d	d	(-)	+	+	+	+
Glycine	(-)	-	-	-	-	-	-	-	-	-	-	+	+	+
L-arginine	+	+	+	-	-	-	-	-	(-)	-	+	+	+	+
L-histidine	(+)	+	(+)	-	-	-	-	-	-	-	+	+	(+)	+
D-galactose	d	(-)	(-)	-	d	-	-	-	d	-	+	(+)	d	+
D-xylose	-	-	(-)	(-)	(-)	(-)	-	-	(-)	-	-	+	(+)	+
D-ribose	+	+	+	-	-	-	(-)	(-)	(-)	-	(-)	+	+	+
L-arabinose	-	-	(-)	-	(-)	-	-	-	(-)	-	-	(+)	d	+
Xylitol	+	+	(+)	-	-	-	-	-	-	-	-	(+)	-	+
D-mannitol	+	+	+	(-)	d	(-)	d	d	d	-	+	+	(+)	+
Hydrolysis of:														
Milk (50% v/v)	-	-	-	(-)	(-)	(-)	-	-	-	-	d	+	+	+
Tyrosine	(+)	(+)	(-)	-	-	-	-	-	-	-	-	+	+	+
Xanthine	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Acid from:														
Sucrose	-	-	-	d	(+)	d	-	-	-	d	-	-	-	-
Lactose	-	-	-	(-)	d	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	(-)	-	-	-	-	-	-	-	-	-

Table 5.3.1 Characteristics diagnostic for the identification of coryneform clusters.

Scoring: + = 80-100%; (+) = 67-79%; d = 34-66%; (-) = 21-33%; - = 0-20%

Clusters: A1 and A2 = Nocardia; B1 = Cellulomonas; B2 = Microbacterium, Corynebacterium or Curtobacterium; C1 and C2 = Rhodococcus; C3 = Mycobacterium; D = Curtobacterium; E1 = of type Brevibacterium protophormiae; E2 = of type Arthrobacter globiformis. Adapted from Sieler et al. (1980), Sieler et al. (1977)

Utilization of carbon sources, hydrolysis and acidproduction were carried out on plates. Each test was performed three times, inoculated with acetate-grown cultures (Table 5.3.2).

Culture	MVAm	MVAe	MVA/2	EV1	E1/3	MWD	M4/20	M1/5S
Utilization of:								
Pyruvate	+	+	+	+	+	-	+	+
DL-lactate	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	+	+	+
5-amino-valerate	+	+	+	+	+	+	+	+
DL-malate	-	-	-	-	-	-	-	-
Succinate	+	+	+	+	+	+	+	+
Citrate	-	+	+	+	+	+	+	-
4-hydroxybenzoate	+	+	+	+	+	+	+	+
L-aspartate	-	-	+	+	-	+	-	-
Glycine	-	-	+	+	-	-	+	-
L-arginine	+	+	+	+	-	-	-	+
L-histidine	+	+	+	+	+	-	-	+
D-galactose	-	-	-	+	+	-	-	-
D-xylose	+	+	+	+	+	+	+	+
D-ribose	+	+	+	+	-	-	-	+
L-arabinose	-	-	-	+	+	+	-	-
Xylitol	+	+	+	+	-	-	-	+
D-mannitol	+	+	+	+	+	-	+	+
Hydrolysis of:								
Milk (50% v/v)	+	+	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	+
Xanthine	+	+	+	+	+	+	+	+
Acid from:								
Sucrose	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-

Table 5.3.2 Characteristics of coryneform isolates from the Harfoot collection.

The test strain results and the clusters were compared using a 1-2-3 spreadsheet software (Lotus Development Corporation, USA). The full spreadsheet (3082 cells) is not shown. The major logical function @IF (cond, x, y) (i.e. Use "x" if the condition "cond" is true, use "y" if the condition "cond" is false.) was used as the basis for the following cell manipulator:

@IF((+R=+T),(@IF((+R=100),1,0.5)),((@IF((+R<+T),(+R/+T),(@IF((+R=25),0.25,0)))))

Where "R" is the cluster % positive (0, 25, 50, 75 or 100) and T is the test result (100 or 0). The manipulator was designed to fulfil the following functions:

Cluster % positive	Test Result	Value
80-100	+	1.00
67-79	+	0.75
34-66	+	0.50
21-33	+	0.25
0-20	+	0.00
80-100	-	0.00
67-79	-	0.00
34-66	-	0.00
21-33	-	0.25
0-20	-	0.50

Table 5.3.3 Values assigned to possible combinations of test results and % positive figures.

Thus a value of between 0 and 1 was assigned to each result compared to each of the 14 clusters, giving more weight to positive matches than negative. A simple matching coefficient between test strains and the clusters suggested by Sieler were calculated:

$$\text{Coefficient} = \frac{a + q(b)}{n} \times 100$$

Where: a = positive matches

b = negative matches

q = a variable factor

n = number of tests

The q factor is used to describe the unequal weighting of positive to negative matches described above.

The simple matching coefficients for the strains and the clusters (Table 5.3.4) suggest that the majority of the cultures group into clusters E, E1 and E2.

Cluster	MVAm	MVAe	MVA2	EV1	E1/3	MWD	M4/20	M1/5S
A	59.78	64.13	*68.48*	*68.48*	51.09	41.30	47.83	59.78
A1	*63.04*	67.39	*69.57*	*67.39*	52.17	43.48	47.83	59.70
A2	53.26	56.52	58.70	58.70	44.57	38.04	39.13	53.26
B	21.74	19.57	15.22	10.87	21.74	21.74	26.09	21.74
B1	18.48	16.30	14.31	16.30	25.00	20.65	22.83	18.48
B2	22.83	20.65	16.30	11.96	22.83	22.83	27.17	22.83
C	31.52	35.81	35.87	33.70	38.04	36.96	40.22	31.52
C1	33.70	38.04	38.04	33.70	38.04	38.04	42.39	33.70
C2	34.78	38.04	38.04	40.22	42.39	38.04	40.22	34.78
C3	23.91	27.17	25.00	20.65	29.35	33.70	33.70	23.91
D	36.96	36.96	39.13	41.30	36.96	26.09	28.26	36.96
E	*65.22*	*69.57*	*78.26*	*84.78*	*67.39*	55.43	*60.87*	*65.22*
E1	*64.13*	*68.48*	*77.17*	*81.52*	*66.30*	**57.61**	**63.04**	*64.13*
E2	**67.39**	**71.74**	**80.43**	**89.13**	**67.39**	54.35	58.70	**67.39**

Table 5.3.4 Simple matching coefficients between Gram positive Harfoot cultures and the eight clusters suggested by Sieler et al. (1980). Figures in bold are the highest values for each culture. Results above 60% are in italics.

From these results, the following putative classifications could be made: MVAm, MVAe, MVA2, EV1, E1/3, M4/20 and M1/5S all group with cluster E. Reference strains for this group include *Brevibacterium* and *Arthrobacter* strains. Culture MWD seems unsuited for identification by this method. Further tests would have to be carried out to make the

classification more specific, particularly an examination of the peptidoglycan composition of cell walls.

No further experiments were carried out to elucidate the cultures identity as the plates used in the above tests (particularly those with amino acids as substrates) revealed that all the cultures were mixed consortia of organisms. The bacteria of the individual consortia were identical in size and shape and were only distinguished by colony morphology. The first slopes made by Dr Harfoot were checked and these yielded the same result. This, of course, removes any possibility of making valid conclusions from the above taxonomy. Further experiments were performed in an effort to separate the bacteria in the consortia responsible for the primary cleavage of compounds such as DES and DMS.

5.4 The metabolism of diethyl sulphide

Previous sections in this chapter and others have described some of the evidence that led to the hypothesis that the Harfoot cultures were all bacterial consortia. These include variable growth of MVA on CS_2 (Chapter 4, section 2.1), apparently dependent on the growth substrate of the inoculant, and colony dimorphism when all cultures were grown with amino acids as sole carbon and energy source.

The experiment that conclusively showed the presence of more than one bacterial type was originally designed to gain more information on the enhanced growth of the Harfoot cultures on DES when flasks were left stationary rather than shaken.

This characteristic was thought to be attributable to the organisms' microaerophilic rather than aerobic growth. It was thought that some idea of the organisms' oxygen tolerance could be gained from monitoring growth in seeded soft agar contained in test tubes. Representatives of each morphological type were grown on 5 mM acetate and inoculated into 0.7% agar. Each organism was tested against several substrates (see table 5.4.1).

Culture	MVAm	MVAe	EV1	D4	TF1	TF2
Medium:	Depth of band of growth (mm)					
Minimal medium + DES	4/0	3-5/0	3-9/0	6/0	4-8/0	3-6/0
TvM	3	3	6	7	4-5	5
TvM + DES	3	4	6	7	4-5	5
Nutrient Agar	0	0	0	0	0	0

Table 5.4.1 Depths of 0.7% agar at which selected Harfoot cultures form visible bands of growth. "/" denotes were more than one band of growth is present.

It was assumed that no growth was entirely anaerobic, after the work of Nelson (1989) and Nelson and Jannasch (1983), but that oxygen was present throughout the medium, decreasing in concentration with depth.

When the organisms were seeded in nutrient agar, growth was restricted to the very top of the test tube, and did not penetrate into the agar. However, if DES or thiosulphate were used as growth substrates, bands of bacterial mass formed deeper in the agar, with no growth on the surface.

This could have meant that the cultures had different oxygen demands under different growth conditions, much as *Beggiatoa* will favour different oxygen concentrations according to the amount of sulphide present (Nelson and Jannasch, 1983). This was countered by an experiment in which MVAm was repeatedly sub-cultured on nutrient agar. The bacteria lost the ability to use DES as sole carbon and energy source after four sub-cultures, showing that some factor had been removed. A control of MVAm sub-cultured on acetate yielded cells able to use DES, so it was unlikely that the factor removed was genetic in origin, such as a plasmid.

The organisms found growing at reduced oxygen tension in TvM or MinC with DES were found to be of a thiobacillus type. Bacilloid cells staining Gram negative could be found in the relevant area of the agar with the aid of a microscope. Attempts to isolate the organisms failed (see chapter 2, section 2.4). No growth could be detected in flasks, degassed with nitrogen, containing TvM or MinC + DES with oxygen at 1.6, 3.2, 4.8, or 6.4% (v/v).

A hypothetical model for the consortias' survival on DES and acetate can be constructed (see figure 5.4.1)

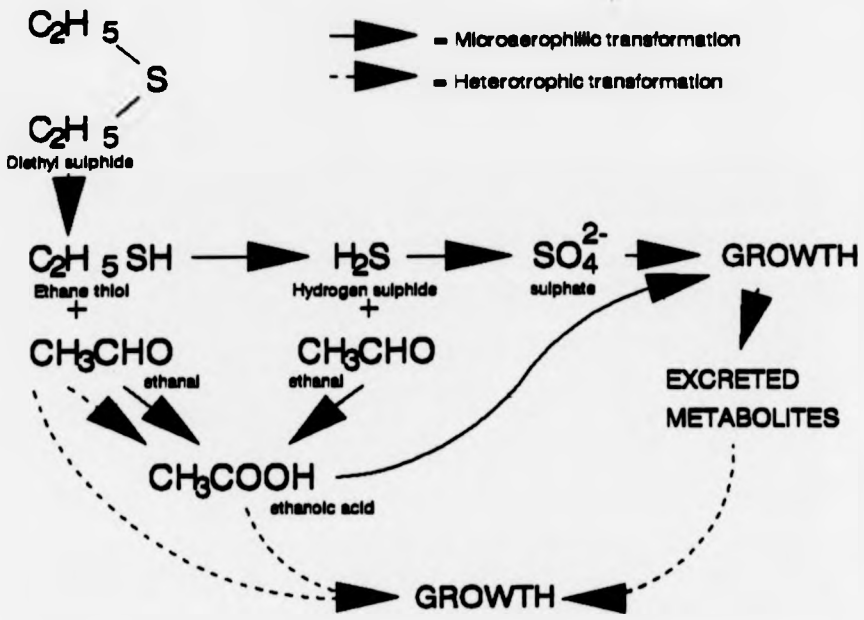


Figure 5.4.1 A hypothetical pathway for the metabolism of DES by mixed cultures.

The microaerophilic thiobacilli perform the primary cleavage of DES, and ultimately gain energy from the oxidation of the sulphide released, in much the same way as *Thiobacillus thioparus* E6 grows on DMS (Smith and Kelly 1988a). However, their metabolism of DES results in acetate or ethanal being released from the cell. These compounds could provide the aerobic part of the consortium with a growth substrate.

This theorem explains many of the results. Both microaerophile and aerobe survive on the acetate agar used to maintain the culture collection, as the both organisms can use acetate. Perhaps the aerobe might have some effect on lessening local oxygen concentrations, by using up O_2

during its own growth, providing better conditions for the microaerophile. When a culture is inoculated onto nutrient agar, only the aerobe grows, and eventually the ability to use DES is lost.

Growth on any sulphur compound would be dependent on the microaerophilic "thiobacillus". The variable growth of MVA on CS_2 could have been accounted for by differences in the physiological state of the microaerophile, or how many of these organisms were present in the first inoculum. Similarly no growth on thiosulphate was reported for the culture MVA under aerobic conditions, but growth does occur under soft agar. If the cultures are grown in DES soft agar, the microaerophiles appear below the surface, as with thiosulphate, but also support the growth of the aerobes at the top of the agar. Enhanced growth in stationary liquid cultures containing DES is a result of the conditions favouring better metabolism of DES by the "thiobacilli".

Although it was not possible in the time available to isolate the microaerophilic "thiobacillus", the concept of a thiobacillus supporting the growth of a coryneform aerobic bacterium can be demonstrated (see figure 5.4.2). The *Rhodococcus* species strain TTD-1 will not grow in the presence of DES, while *Thiobacillus thioparus* will oxidise this compound. Introduction of a suspension of *T. thioparus* and 1 mM DES into the left hand chamber of the apparatus resulted in an increase in optical density of a culture of TTD-1, separated into the right hand side of the apparatus by a 0.2 μm nitrocellulose filter. The only way TTD-1 could

have grown was on the excreted products from the oxidation of DES by *T. thioparus*.

The Harfoot culture collection was thus composed of mixed cultures. In each case, whether the dominant bacterium was Gram positive or negative, the organism responsible for the primary degradation of DES, and possibly other sulphides, was a gram negative rod with the capability of growing on thiosulphate microaerophilically.

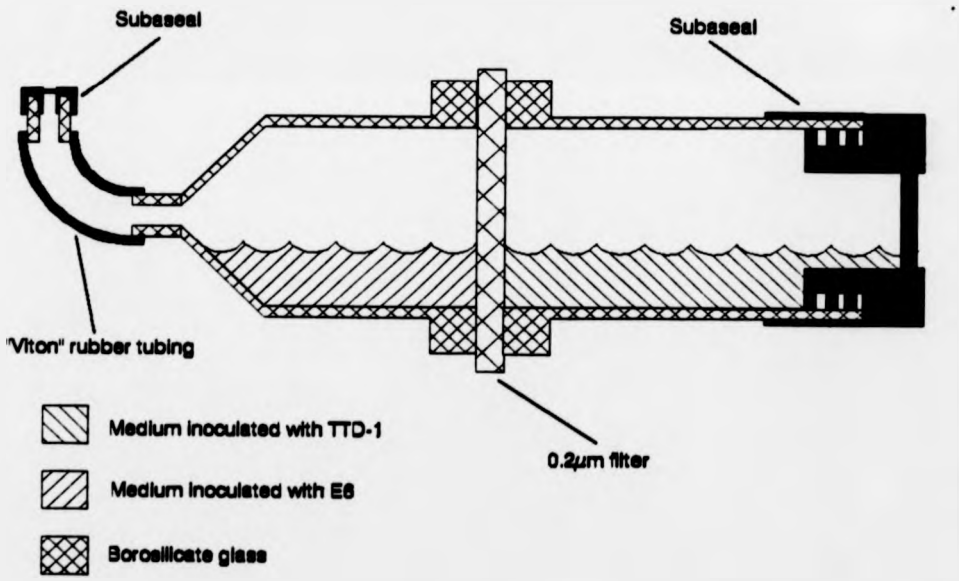
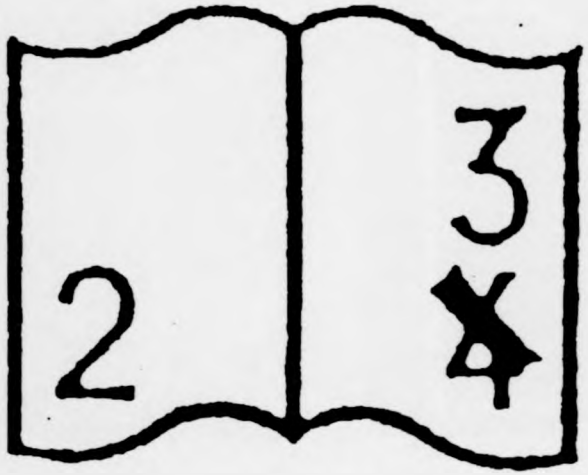


Figure 5.4.2 Apparatus to demonstrate Thiobacillus thioparus supporting growth of Rhodococcus TTD-1.

PAGINATION ERROR



CHAPTER 6:
METHYLOTROPHIC GROWTH ON SODIUM METHANE SULPHONATE

6.1 Introduction

Little has appeared in the literature on methane sulphonate (MSA) in relation to biological systems. The data available are restricted to the use of MSA as a sulphur source for two organisms. Work on *Chlorella fusca* by Biedlingmaier and Schmidt (1983) and then Krauss and Schmidt (1987) showed that of C_1 to C_8 -*n*-alkyl sulphonates, MSA was the least suitable sulphur source for the alga. Earlier, Cook and Hütter (1982) described a Gram negative bacterium (strain 26) capable of using "methylsulphonate", again as a sulphur source. The bacterium used acetate or glucose as a carbon source. The organism was originally isolated to demonstrate desulphurization of ametryne (a heterocyclic herbicide, see figure 6.1.1), and hypothesized that MSA could be an intermediate in metabolism.

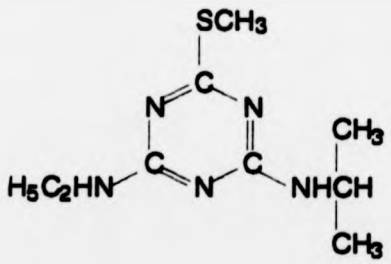


Figure 6.1.1 Molecular structure of Ametryne, a heterocyclic herbicide.

However, only strain 26 could use MSA as a sulphur source, whereas other bacteria capable of degrading ametryne, and its derivative prometryne, could not. The methyl group of MSA was not released as methane in strain 26.

Bacteria have been shown to use the phosphate analogue of MSA, methane phosphonate (MPA). This is degraded by bacteria such as *Pseudomonas testosteroni* and *Escherichia coli*, with the release of methane (Daughten *et al*, 1979a, 1979b; Cook *et al.*, 1979; Ghisalba *et al.*, 1986).

No organism has been isolated with the capability to derive carbon and energy from MSA, despite the entry of the compound to the natural environment for at least ten thousand years (Saigne and Legrand, 1987). It seemed unreasonable to suppose that this stable organic acid persisted in the soil. Indeed, alkane sulphonates are known to be biodegradable. *Pseudomonas* species were readily isolated from garden soil and surface water with alkane sulphonates (C_4-C_{12}) as their only source of carbon and energy, releasing an aldehyde and sulphite (Thysse and Wanders, 1972, 1974).

In enrichment culture, MSA could have been considered a carbon source suitable for methylotrophic growth, with oxidation of the methyl group providing energy. Chemolithotrophic growth, with cleavage of the C-S bond and the oxidation of released sulphite providing energy, would also have been possible. Thus an enrichment culture could have yielded restricted methylotrophs, facultative methylotrophs or *Thiobacillus*-type organisms. Another possibility was that MSA could only be used as a sulphur source.

Biogeochemical data suggested that MSA was present

throughout the environment (See section 1.4), and so an enrichment from garden soil was chosen.

6.2 Enrichment and isolation of methane sulphonate users

The low phosphate medium (MinE) chosen for the enrichment of bacteria capable of using MSA as sole carbon and energy source was that of Owens and Keddie (1969). Originally developed for the isolation of coryneform bacteria, it was adapted by L.J. Zatman (University of Reading) in later years to serve as a medium for the isolation of methazotrophs (i.e. those bacteria using MMA as sole carbon, energy and nitrogen source). Since MinE supported the growth of a wide variety of facultative methylotrophs as well as more conventional heterotrophs, it was once again adapted, to maximise the recovery of MSA-users.

In order to introduce some degree of selectivity in the properties of the medium, sulphur-containing compounds, such as magnesium sulphate, were omitted (see composition of MinE and MinE-S in Sections 2.2.6.1 and 2.2.6.2 respectively). These were replaced by the chloride form of the compound, in an effort to maintain the ionic balance of the medium. This policy was not extended to the trace metal or vitamin solutions, which had a very low concentration of sulphate. It was hoped that by limiting the sulphur available, conditions would favour those organisms capable of using MSA

as sulphur and/or carbon source. A measure of the effectiveness of this approach can be gained from the observation that conventional methylotrophs, such as *Bacillus* PM6, would not grow in the adapted medium even in the presence of MMA, that organism's isolation substrate.

Attempts by second year undergraduates (at the University of Warwick) to isolate MSA-users using NMS (see Chapter 2, section 2.2.5.3) failed. Similarly, the number of MSA-users isolated from MinE enrichment and purification culture was less than that from MinE-S (Murrell, J.C. and others, University of Warwick, unpublished results.). This would seem to indicate that the choice of MinE and the omission of sulphur provided an almost ideal medium for the isolation of MSA-users.

Inoculating soil and water samples into media containing MSA as sole carbon and energy source did not give rise to organisms able to grow on MSA. Thus it was decided to use a chemostat-enrichment procedure with both methylamine hydrochloride (MMA) and MSA. This would have enhanced recovery of methylotrophic bacteria that might also have used MSA as a sole or co-substrate, and would have also allowed enrichment of specialist MSA-users unable to use MMA, assuming that these organisms could have competed successfully with other organisms. Such procedures have been successful in isolation and competition experiments with sulphur-using bacteria (Smith and Kelly, 1979; Kelly and Kuenen, 1984)

The decision to add MMA was also influenced by the possibility that MSA could only act as a sulphur source for bacteria (see above). If this was indeed the case, then methylotrophs using MSA as sole sulphur source would have been isolated.

Since no bacteria had previously been isolated using MSA, the potential toxicity of the sulphonate was unknown. In preparing media for the enrichment, the concentration of carbon as MMA and MSA was limited to 10 mM (7 mM MSA + 3 mM MMA), as this was a carbon source concentration used for the growth of known methylotrophs.

The chemostat was inoculated with 100 g of garden soil, taken from under a rose bush. The proximity to a rhizosphere would have encouraged a diversity of organisms to grow or survive in the soil. The chemostat equipment was used as a batch culture for the first few weeks. After this time fresh medium was fed in for one hour in every three and then continuously. Five weeks after inoculation, the enrichment was visibly turbid and it was decided to take a sample and serially dilute it, plating the dilutions onto various agars (nutrient agar, MinE + 10 mM MMA and MinE + 10 mM MSA). Organisms showing seven different colony forms were subcultured from these plates. Nine cultures were obtained from these, growing on MSA as sole carbon and energy source. They were named M1-M9.

6.3 Characterisation of Strains M1-M9

6.3.1 Morphology

The morphologies of the bacteria isolated capable of using MSA as sole carbon and energy source are listed in table 6.3.1.1.

Strain Number	Morphology	Gram stain	Origin
M1	Rod, single	-	Chemostat
M2	Rod, single/pairs	-	Chemostat
M3	Rod, single	+	Chemostat
M4	Rod, single	-	Chemostat
M5	Slender rod	+	Chemostat
M6	Rod, single	-	Chemostat
M7	Rod, single	-	Chemostat
M8	Coccus	+	Chemostat
M9	Rod, single	-	Chemostat
M56	Rod, single	-	Aerial

Table 6.3.1.1 The morphologies of the MSA users M1-M9 and M56. M56 was an aerial contaminant isolated from a MinE-S plate

6.3.2 Selection of M2 for further study

Although M1 and M3-M8 were purified to single colonial and morphological types, their colonies were small (<1 mm) and often difficult to see on MSA agar plates. Moreover, growth in liquid culture (with MSA as sole substrate) took 7 days or more to reach visible turbidity. However, the isolate M2 grew as white colonies, 1-2 mm in diameter, on MSA agar and produced visibly turbid MSA liquid cultures within three days. Quite evidently M2 had a far better yield on MSA than any of the other isolates and so was more suited to further biochemical and microbiological study. Furthermore, M1 and M3-M9 did not survive repeated subculture on agar and so

stocks of these bacteria were limited to the primary subcultures, which were eventually unusable due to age. The remainder of this thesis describes the properties of M2, a short Gram negative rod growing singly or in pairs, in comparison with other methylotrophs.

6.3.3 Attempted Identification of the strain M2

An axenic culture of M2 contained rod-shaped cells, approximately 1 μm in diameter and 2-4 μm in length, appearing singly or in pairs. No pleiomorphism was noted on any of the media used. No refractile bodies could be seen under the phase contrast or light microscope. The resting stages remain unknown. The bacteria appear to be highly motile when viewed under the light microscope. Gram reaction is negative, but the cell wall ultrastructure has yet to be checked. Cultures are aerobic, but can grow under slightly reduced oxygen tension, with a growth temperature optimum of 30 °C. It will not grow on common laboratory media such as nutrient agar, and has a requirement for a low phosphate medium.

To gain some insight into the genus of this Gram negative rod, the API20E system was used (see section 2.8.1). The results from this system led to an identification of the organism as *Pseudomonas paucimobilis*. However, this identification was based on few positive results - 4 from a possible maximum of 32. Of these results, two tested for the presence of enzymes in suspensions of cells (urease and catalase) and one was that M2 was motile. The results from

the API system differed from those obtained elsewhere, Eg sugar utilization. This was probably due to the use of the standard API medium, a medium far richer than the MinE media normally used for growth of M2. The API medium contained yeast extract and an unidentified compound listed as "mineral base". Although M2 will not grow on rich media such as nutrient agar (NA), the API system relies on the tests being standard for whatever bacterium used, so the API medium was obligatory for this experiment. It was therefore unsurprising that growth was obtained in only one test. Given the number of organisms whose growth is inhibited by yeast extract, the results obtained could equally be applied to any bacterium with similarly restricted growth conditions.

The final failing of the API20E system is that the database used for identification is weighted towards medically isolated bacteria, and contains few from non-human sources. Indeed, when a type strain of *P. paucimobilis* was obtained, it failed to grow under the same conditions as M2, and could not sustain growth on any C_1 compound. It thus seems unlikely that M2 is related to the species. The restricted composition of the media that M2 will grow on preclude the strain's inclusion into the genus *Pseudomonas*, and so this genus cannot be used in its function as a repository for incompletely identified methylotrophs (Eg *Pseudomonas* M27, *Pseudomonas* MS, *Pseudomonas aminovorans* etc).

Characteristic	*Methylo. glygogenes*	*Methylophilus methylotrophus*	M2
RuMP cycle for carbon assimilation	+	+	-
Acid from: D-glucose	-	+/-	-
D-fructose	-	NA	-
Growth on: D-glucose	-	+	+
Sucrose	-	-	+
Glycerol	-	-	+
Succinate	-	-	+
Acetate	-	-	+
Fructose	-	+	+
MMA	+	+	+
Formate	-	+	+
H_2S formation	-	-	-
Urease	+	NA	+
Oxidase	+	+	-
Catalase	+	+	+
Growth at 30 °C	+	+	+
at 37 °C	+/-	+	+
at 45 °C	-	+	-

Table 6.3.3.2 Characteristics of Methylobacillus glycogenes (Methylo. glycogenes), Methylophilus methylotrophus and isolate M2, selected to show their dissimilarities. NA = data not available, +/- = strain dependent characteristic.

Dr P. Green of the National Collection of Industrial and Marine Bacteria Ltd. kindly agreed to attempt an identification, but could only suggest that the bacterium was of the type *Methylobacillus* or *Methylophilus*. These genera differed markedly from M2 (See table 6.3.3.2) and the suggestion seems to have little basis in fact. M2 uses the serine pathway for carbon assimilation from C_1 compounds, whereas *Methylobacillus* and *Methylotrophus* use the RuMP pathway. M2 could be described as a facultative methylotroph, growing on many poly carbon compounds including glucose, sucrose and acetate. *Methylobacillus* and *Methylotrophus* are described as obligate methylotrophs

(Anthony, 1982) showing no growth with any non-C_1 compound, except fructose in the case of *Methylophilus methylotrophus*. Although it can be said that biochemical tests form no basis for classification, such diverse differences would surely be reflected in the genomic composition of M2, *Methylobacillus* species and *Methylotrophus* species. It thus does not seem unreasonable to suppose that M2 represents at least a new species of methylotroph, more similar to serine-pathway facultative methylotrophs such as *Methylobacterium extorquens* AM1, *Pseudomonas* M27 or *Pseudomonas* MS.

6.3.4 Growth substrates of the isolate M2

The growth substrate range (see table 6.3.4.1) differs from previously published results and would seem to confirm the hypothesis that M2 represents a new type of methylotroph.

The ability of M2 to use both C_1- and poly-carbon compounds such as sucrose means that the organism can be best described as a facultative methylotroph. Extending Zatman's terminology (Zatman, 1981), M2 could also be described as a methylothiotroph, using MSA as sole carbon, energy and sulphur source, much as the methazotroph *Methylobacterium extorquens* can use methylamine as sole carbon, energy and nitrogen source. It will not be possible to name the organism until such time as a full classification is completed.

Substrate	Methylotroph Strain			
	MS	AM1	M27	M2
Sodium methane sulphonate	-	-	NT	+
Methane	-	-	-	-
Methanol	-	+	+	+
Formaldehyde	-	-	-	+
Sodium formate	-	+	+	+
Hydrogen and CO_2	NT	-	NT	-
Serine	+	-	-	+
Methylamine hydrochloride	+	+	+	+
Dimethylamine hydrochloride	+	+	-	+
Trimethylamnie hydrochloride	+	+	NT	+
Trimethyl sulphonium chloride	+	-	-	+
Nitromethane	NT	NT	NT	+
Methane phosphonate	-	NT	NT	-
Dimethyl sulphide	-	NT	NT	-
Dimethyl disulphide	-	NT	NT	-
Carbonyl sulphide	NT	NT	NT	-
Carbon disulphide	NT	NT	NT	-
Sodium aminomethane sulphonate	NT	NT	NT	+
Methyl methane sulphonic acid	-	NT	NT	-
Monomethyl sulphate	NT	NT	NT	-
Acetamide	NT	NT	NT	-
Thioacetamide	+/-	NT	NT	+
Ethanol	-	+	+	-
Glycine	-	-	-	-
Alanine	+	-	NT	+
Glucose	+	+	+	+
Fructose	+	+	NT	+
Sucrose	NT	NT	NT	+
Sodium acetate	+	-	+	+
Sodium propionate	+	-	+	+
Sodium lactate	+	+	+	+
Sodium pyruvate	+	NT	+	+
Sodium glutamate	+	+	NT	+
Sodium citrate	-	+	+	-
Sodium benzene sulphonate	NT	NT	NT	-

Table 6.3.4.1 The use of compounds as sole source of carbon and energy by several methylotrophs. + = an increase in biomass in the sole presence of the compound. MS = Pseudomonas strain MS (Kung and Wagner (1970); AM1 = Methylobacterium extorquens strain AM1 (Peel and Quayle, 1961); M27 = Pseudomonas strain M27; M2 = Methylotrophic isolate M2 (Baker et al. 1991); NT = Not tested. Data for MS, AM1 and M27 from Wagner and Quayle (1972), supplemented with original data.

6.3.5 Antibiotic Resistance of M2

Using compounds impregnated into "Mastrings", the following antibiotics were tested for the inhibition of growth of a lawn of M2 spread on agar plates containing MSA or formate as sole source of carbon and energy (Table 6.3.5). The zones of inhibition observed were the same whether the organism was grown on MSA or formate.

Antibiotic	Amount (per disc)	Diameter of zone of inhibition
Ampicillin	2 μg	0
Cephalondine	5 μg	0
Chloramphenicol	25 μg	0
Clindamycin	2 μg	0
Cloxacillin	5 μg	0
Erythromycin	5 μg	0
Fusidic acid	10 μg	0
Gentamicin	10 μg	10 mm
Lincomycin	2 μg	0
Methicillin	10 μg	0
Novobiocin	5 μg	0
Penicillin G	1 unit	0
Streptomycin	10 μg	10 mm
Sulphamethoxazole	25 μg	0
Tetracycline	10 μg	3.2 cm
Trimethoprim	1.25 μg	0

Table 6.3.5 Inhibition of formate- and MSA-grown plate cultures of M2 by antibiotics.

6.3.6 The %G+C content of DNA extracted from M2

Two methods of DNA extraction were used, each routinely used by different workers (section 2.9.12), one involving the removal of unwanted matter from the DNA mainly via the use of cesium chloride gradient centrifugation, the other relying on enzymatic digestion and precipitation in cold ethanol to achieve the same effect. The former produced DNA of a higher quality, while the latter produced a pink

pigmented substance instead of DNA. Neither method produced DNA of sufficient purity to enable the calculation of %G+C.

The methods of DNA extraction used were fruitless because of the white, stringy matter that the cells released on lysis. This effectively adhered DNA, cell wall fragments and compounds to each other, making separation an inexact task. The DNA obtained from the cesium chloride method was used in a determination of %G+C by a thermal melting method, monitoring the absorbance of the DNA at 260 nm and 280 nm over temperatures between 25 and 110 °C. For pure DNA, the helix unwinds as the temperature rises. This causes a corresponding rise in OD_{260} and OD_{280}, as absorbance is a colligative property. In the case of DNA isolated from M2, the impurities bound the DNA together until the temperature had risen to a point where the macromolecule did not merely separate, but degraded altogether.

6.4 Growth of other bacteria on MSA

6.4.1. Introduction

After the ease of isolating an MSA user from the natural environment, the hypothesis that MSA degradation is a prevalent feature of many bacteria capable of growing on other C_1 compounds was formulated. This was tested using methylotrophs and some bacterial laboratory strains (see table 2.1.1)

6.4.2 Growth in the presence of MSA alone.

A variety of cultures was tested for their ability to grow

on MSA as sole carbon and energy source. This ability was assessed by supplementing the organism's isolation medium with 5, 10 and 15 mM MSA in solid and liquid culture, though in the case of *T. versutus* further chemostat and oxygen electrode work was also carried out (see below). None of the cultures listed in table 2.1.1 showed any increase in biomass in the presence of MSA. Similar experiments resulted in the conclusion that the bacteria in the Harfoot culture collection (Table 2.1.2) would grow with MSA as sole carbon and energy source.

6.4.3 Mixotrophic Growth

In addition to testing the culture collection on MSA alone, in each case a growth curve was prepared where MSA supplemented another carbon source. This was to assess if the test organism could cometabolise MSA or showed any growth inhibition by it. These tests cannot be considered to be complete as few growth parameters were varied, and under different conditions this cometabolism may occur in the same strains. An example, the growth of PM6 on MMA in the presence and absence of MSA, is shown in figure 6.4.3.1. The only effect increasing MSA concentration had was to increase the lag time of the culture.

Although none of the cultures tested would grow with MSA as sole carbon and energy source, MSA did not have any inhibitory effect on the organisms' growth on other substrates, apart from increasing lag times during batch culture. It could be argued that this is a reflection of the

presence of MSA throughout the natural environment. It is possible that many of the cultures could use MSA as a sulphur source, though without a suitable assay for MSA this would be harder to demonstrate. The use of MSA as a sulphur source has been demonstrated for a *Pseudomonas*-type organisms (Cook and Hütter 1982).

Figure 6.4.3.1 The growth of Bacillus strain PM6 on methylamine (MMA) and in the presence of sodium methane sulphonate (MSA). The optical densities of the cultures had not altered from that measured at 77.5 hours when readings were taken five days later.

6.4.4 Thiobacillus versutus.

The well-characterised thiobacillus, *T. versutus*, was selected as a bacterium with the possible capability of using MSA. It possesses biochemical pathways for the assimilation of both C_1- and sulphur-compounds (Taylor and

Hoare, 1969; Wood and Kelly, 1977), giving it the capability of growing on a plethora of substrates including thiosulphate, methanol, methylamine and glucose. Since MSA is a methyl group attached to a sulphonate group, it was thought that *T. versutus* might have the necessary enzymes for the compound's complete oxidation. In this model the organism would have gained energy from the oxidation of the methyl group to CO_2, and carbon from the fixation of CO_2 via RUBISCO.

6.4.4.1 The effect of MSA on chemostat-grown Thiobacillus versutus.

A 750 ml chemostat was established as described in *Materials and Methods* (Chapter 2, section 2.7.4). In continuous culture it was grown on MMA alone or MMA and thiosulphate. When 10 mM MSA was introduced into the medium as a supplement, the optical density of the culture decreased and eventually the organism was washed out of the chemostat, whether the organism was growing on single or dual substrates.

6.4.4.2 The effect of MSA on the oxidation of thiosulphate by Thiobacillus versutus.

Cells from a thiosulphate limited chemostat were harvested and resuspended in a volume of phosphate buffer approximately one third of the sample size, to an OD_{440} of 0.441. Thiosulphate (50 mM) was added after the cell suspension had equlibrated for five minutes. Methane

sulphonate (10 mM) was added as a 1 M aqueous solution when approximately half the available thiosulphate had been oxidised. The MSA had no effect on the rate of oxidation or the amount of thiosulphate oxidised.

In a second version of the experiment, the culture was pre-incubated with 10 mM MSA for 15 minutes at 30 °C, in the cell of the oxygen electrode. After this time, 50 mM thiosulphate was introduced into the cell. Once again the MSA had no effect on the rate of oxidation or the amount of thiosulphate oxidised.

Similar experiments yielded the same result with *Thiobacillus thyasiris* (Wood and Kelly, 1989).

6.4.4.3 Conclusions

Sodium methane sulphonate, at a concentration of less than 10 mM, is not an inhibitor of thiosulphate oxidation. A substrate analogue effect therefore cannot account for the washout shown in a thiosulphate limited chemostat. Since MSA neither stimulates or inhibits oxidation of thiosulphate, a tentative conclusion that MSA does not interact at all with the $S_2O_3^{2-}$ oxidizing system can be made, but this would have to be supported by data from cell-free extracts and purified enzyme studies, rather than the whole-cell data presented here.

It was found that MSA did have some effect on *T. versutus*. The rate of endogenous respiration, measured in the oxygen electrode, fell on the addition of 10 mM MSA. It may have

been that the washout effect noted in the chemostat was due to the action of MSA on some other mechanism associated with the growth of *T. versutus*, such as cell division or the manufacture and action of another essential enzyme (It should be noted that closely related compounds such as methyl methane sulphonate and ethyl methane sulphonate are used as mutagens). This unidentified effect would have been difficult to assess in the time available, particularly as MSA had no visible effect on the growth rates of batch cultures of *T. versutus*.

6.5 Discussion

The success of the enrichment and isolation showed that a diversity of organisms able to grow using MSA as sole energy substrate were present in a randomly chosen soil sample to which the only known input of MSA or other one-carbon compounds was from natural sources. The hypothesis that such organisms are ubiquitous and abundant is supported by this result.

Recently, MSA-users have been shown to be present in many habitats, from marine, fresh water and terrestrial environments (J.C. Murrell and others, University of Warwick, unpublished results). Some of these isolates did not have MSA as their primary isolation medium. It is therefore quite likely that some methylotrophs in culture collections are MSA users. A continuation of screening type cultures and isolation of new MSA users might yet confirm if the metabolism of MSA can be used as a taxonomic tool, in

conjunction with other characteristics. Evidently no firm conclusion on the identity of strain M2 can be made without further study restricted to the identification the bacterium (see chapter 9).

The toxic effects of MSA on bacteria are still unknown. The effect of MSA on the methylotrophs tested was not great, but the wash-out of the *T. versutus* chemostat and increased lag times with MSA concentration shown by *Bacillus* PM6 and others suggest that the compound has some harmful properties.

CHAPTER 7:
METHANE SULPHONATE AS A SOLE SOURCE OF CARBON AND ENERGY FOR THE STRAIN M2

7.1 Growth on methane sulphonate

7.1.1 Growth characteristics in batch culture

The strain M2 grew at 30 °C on 15 mM MSA in liquid culture initially at pH 6.8 with a doubling time of approximately 7 hours. Since MinE is a poorly buffered medium, the pH fell to 4.0 within four days of inoculation. To obtain maximum growth in batch culture, batch cultures were either grown in chemostat apparatus with automatic titration, or 10 µl sterile saturated aqueous bromothymol purple was added per 50 ml of medium. When the pH in the flask had dropped sufficiently to turn the indicator yellow, the medium was neutralised by the addition of 1 or 2 M NaOH. Bromothymol purple had no inhibitory effect on the growth of M2.

On MinE agar with 15 mM MSA incubated at 30 °C, single colonies appeared within five days of inoculation. These colonies were white and 1-2 mm in diameter. If bromothymol purple was incorporated into the medium, the colonies were surrounded by a yellow halo approximately 10 mm in diameter.

When growing on MSA, M2 did not have any specific vitamin requirements. The omission of thiamine, nicotinic acid, pyrodoxine, *p*-aminobenzoic acid, riboflavin, pantothenate, cyanocobalamin or biotin from MinE-S media had no effect on the growth rate or yield of M2 on MSA, but omission of vitamin solution altogether increased the lag time of the culture.

Although cultures of M2 were routinely grown on 15 mM MSA,

batch and continuous cultures grew in media containing up to 250 mM MSA. If the concentration of MSA was raised to 500 mM, growth did not occur. The OD_{440} of batch cultures with <250 mM MSA did not rise above 0.9. It appeared that pH became growth limiting before substrate concentration. Thus accurate values for yield and growth rates could not be calculated, and figures quoted throughout this thesis with regard to MSA must be regarded as apparent growth yields.

When an inoculum was mixed thoroughly with molten 0.5% MinE agar containing MSA (held at 45 °C), after the medium had set and was incubated at 30 °C, growth would first appear 5 mm below the surface of the agar, eventually spreading upwards to appear on the surface in three days. Notwithstanding this slight predilection for microaerophily, M2 would not grow anaerobically either with or without nitrate as an electron acceptor. A heavy inoculum, harvested from an exponential phase culture of M2, was introduced into medium (flushed for 20 minutes with nitrogen), held in a gas-tight container. This was incubated at 30 °C with 15 mM MSA for three days, during which time no increase in acidity of the medium was noted, and there was no increase in the optical density of the culture. Growth could not be initiated by the addition of 5 ml of sterile air-saturated water, but only by bubbling at least 20 ml min^{-1} of sterile air through the medium.

Since there is no simple and routine chemical test for MSA, it was difficult to assess whether M2 was using all of the carbon source available. Derivitization of MSA might have

produced a molecule with low enough boiling point for GC, but the molecule could not be quantitatively and safely derivitized by standard methods suggested for alkane sulphonates described in section 2.11.1.

The lack of an assay for MSA meant that a high degree of confidence had to be placed with the manufacturer of MSA with regard to purity of the substrate. The mass spectrometer at Warwick could not be used as it is linked to a gas chromatograph. The boiling point of MSA is above the temperatures used in GC, but the MSA used in this work contained no impurities detectable by GCMS. Fisons Ltd check the purity of MSA solely by titrating the acid against NaOH. If the amount of acid in the sample exceeds 99%, the sodium salt is prepared from the methane sulphonic acid. The Aldrich chemical company use high performance liquid chromatography (HPLC) to check purity of the end product. This would be a far more precise method. The company did not give any details of how the HPLC was used to detect MSA. Most detectors used in HPLC are based around changes in optical density of the liquids passing through them. A simple experiment showed that the presence of MSA in water does not change the optical density of the solution at any wavelength, even in the presence of a ions such as iron, copper, molybdate and vanadate.

The disappearance of MSA from a batch culture could be measured indirectly. The optical density and pH were monitored during the growth of a 750 ml batch culture grown on 15 mM MSA, held in a chemostat pot. Sterile air was

supplied with magnetically induced stirring, but unlike chemostat culture, the batch culture was not automatically titrated. From an initial value of 6.99, the pH fell to 4.47. (Figure 7.1.1.1)

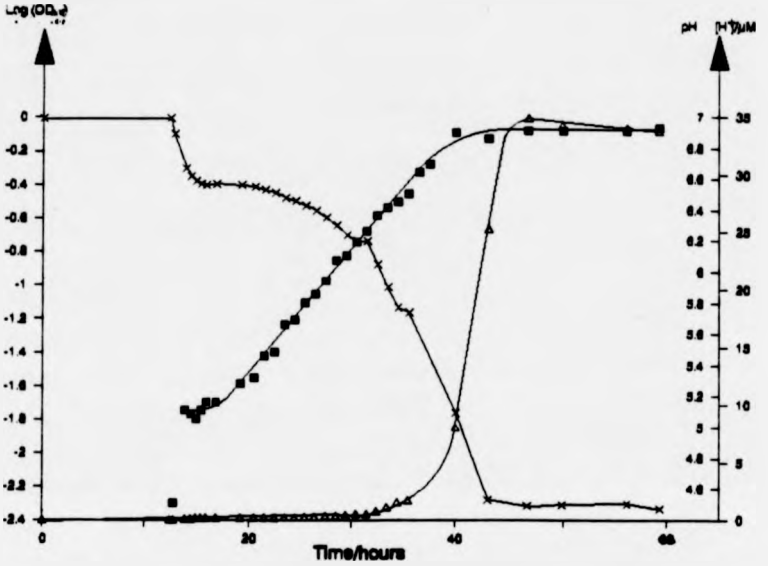


Figure 7.1.1.1 Increase in optical density and change in hydrogen ion concentration in a batch culture of the organism M2. ■ = Optical density measured at 440 nm, × = pH, Δ = concentration of hydrogen ions (μM)

The low buffering capacity of MinE is illustrated by the rapid increase in the concentration of hydrogen ions after 20 hours, when pH had only fallen by 0.4 units. The pH of 4.47 arrested further growth of the culture. The source of this acidity is due to the metabolism of MSA. At least one mole of hydrogen ions are produced per mole of MSA, whether the molecule is completely oxidised in order to produce energy, or oxidised to the level of formaldehyde for assimilation into cell carbon. This is according to the following equation:

$CH_3SO_3Na + O_2 \longrightarrow HCHO + Na^+H^+SO_4^{2-}$

Thus from the graph it can be calculated from the hydrogen ion concentration that at least 33.8 μM MSA were used for the organism to grow to an optical density of 0.856 (205.4 mg dry weight in 0.75 l, calculated from a dry weight determination of 320 mg dry weight $[OD_{440}$ unit$]^{-1}$). This figure for the amount of MSA used does not take into account the pK value (a measure of the buffering capacity) of MinE, and so is an underestimate.

The was MSA supplied to the batch culture at a concentration of 15 mM. Of this a small proportion was ^{14}C-labelled, so that the uninoculated culture had an activity of 11.42×10^6 cpm per millimole MSA as measured by liquid scintillation counting. The ^{14}C activity of the supernatant of a culture spun down at 10,000 g for 10 minutes (figure 7.1.1.2a) gave a representation of the amount of MSA remaining in the medium, assuming that no other ^{14}C-labelled compound was present apart from MSA. The activity in the pellet of spun-down culture was also measured to estimate the amount of ^{14}C fixed.

Activity in the supernatant, of the culture fell at 1529 cpm h^{-1} ml^{-1} during log phase growth of M2. This is equivalent to MSA disappearing at 0.13 μmoles h^{-1} ml^{-1}. This rate was estimated from figure 7.1.1.2a, a graph of the activity in the supernatant and log OD_{440} against time. This would appear to indicate that the disappearance of substrate from the medium was not exponential, as would have been expected,

but when the log of the supernatant activity was plotted against time, little difference can be seen in the linearity of the curve (figure 7.1.1.3). It was possible from this data to conclude that the organism did use MSA for its growth.

By the end of the experiment, when growth had ceased, activity remained, equivalent to 9.38 mmoles MSA. Thus it would appear that the batch culture was pH rather than substrate limited. So, as the optical density reached by the culture was at a maximum of 0.856, then the true yield of M2 on MSA is:

0.856 x 320 = 273.92 mg dry weight per litre
273.92 mg dry weight grown on (15 - 9.38) = 5.62 mmoles MSA
Therefore yield per mole = 273.92 x (1000/5.62) mg
or 48.74 g per mole MSA.

This is approximately four times the value of that observed in batch culture in 250 ml Erlenmayer flasks. It does not, however, take any account of excreted products when calculating the remaining MSA in the medium, which may also be radioactively labelled. Experiments in the oxygen electrode show that M2 accumulates some storage compound (section 7.4.3), which may "leak" from senescent or lysed cells. This would increase the radioactivity in the medium ascribed to MSA. Also, if the activity in supernatants is examined, a rise can be seen between 20 and 25 hours (figure 7.1.1.2), possibly attributable to the excretion of formate from growing cells (section 7.4.4). There is no estimate of

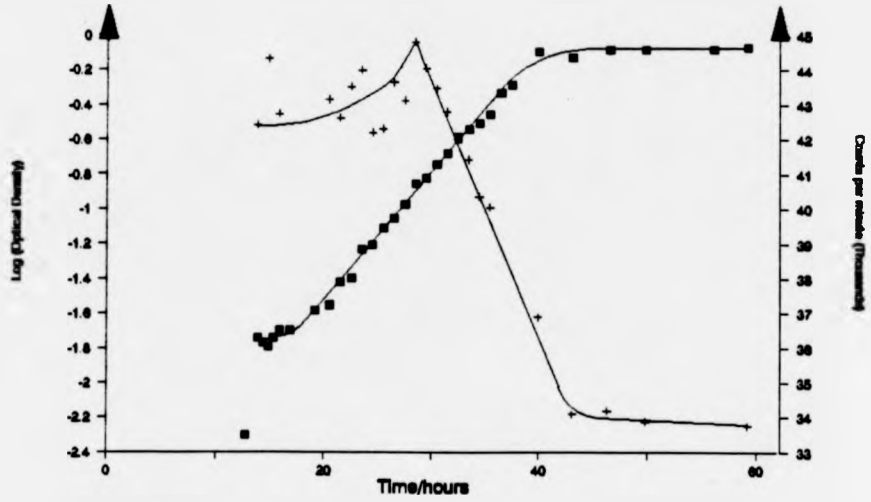


Figure 7.1.1.2a

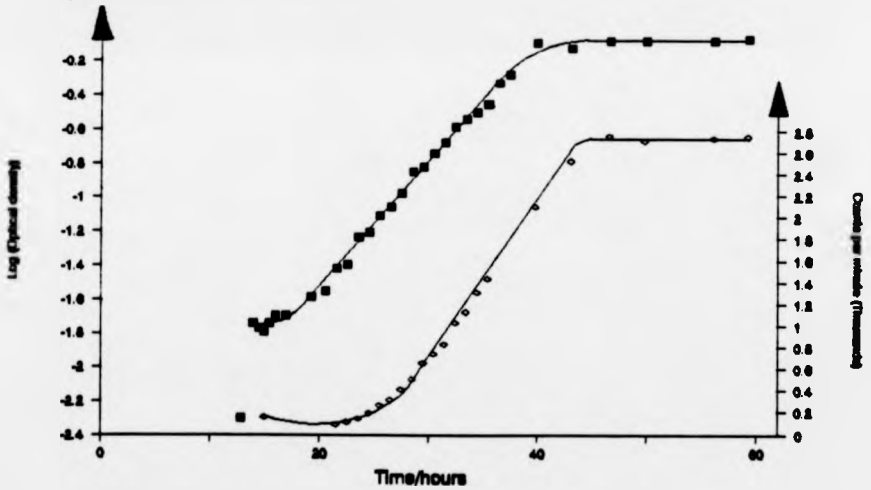


Figure 7.1.1.2b

Figure 7.1.1.2 Changes in the activity of cells and culture supernatants in a batch culture of M2 supplied with 15 mM ^{14}C-labelled MSA. ◇ = activity of cells per 0.5 ml culture sample, + = activity of 0.5 ml culture supernatant, ■ = optical density measured at 440 nm.

formate remaining in the stationary phase culture, so possibly this might also contribute to the apparently high levels of MSA remaining, and the unreasonably high yield calculated from the data.

Since MSA acts as sole source of carbon and energy, the cells remaining at the end of the experiment, did not exhibit activity concomitant with all the MSA being fixed as cell carbon. Some of the ^{14}C carbon escapes as CO_2. Thus the activity of the stationary phase cells is 5563 cpm ml^{-1}. As the dry weight of cells is 320 mg l^{-1} at an

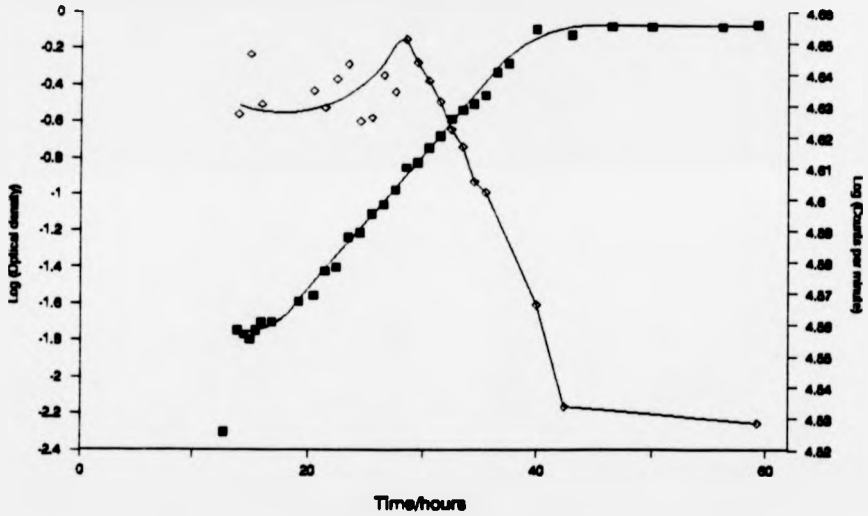


Figure 7.1.1.3 Increase in optical density and change in log (supernatant activity) in a batch culture of the organism M2. ■ = optical density measured at 440 nm, ◇ = log (supernatant activity).

OD_{440} of 1.0, then the amount of cell carbon derived from MSA can be calculated, assuming that the general molecular formula for a bacterial cell is $C_4H_8O_2N$ (van Dijken and Harder, 1975). Taking an average from the cells that were in stationary phase, the percentage of the cell carbon from MSA

was at least 71.4%

7.1.2 The MSA-limited chemostat

With the use of automatic titration, chemostats were limited by MSA as a carbon and energy source, rather than pH and so gave higher yields than their batch equivalents. Continuous cultures maintained at pH 6.6 led to a yield per mole of 17.62 g dry weight, 7.40 g above that obtained in batch culture. Methanol, formaldehyde, formate and sulphite could not be detected in culture supernatants.

The chemostat limited by MSA exhibited a dependence of yield on growth rate, in that the OD_{440} of the culture rose linearly with dilution rate. Above a dilution rate of approximately 0.1 h^{-1}, the chemostat started to wash out. Since maintenance of a constant OD_{440} at a specific dilution rate (D) is dependent on the same number of organisms doubling in the time it takes for that number to flow into the waste pot, the maximum growth rate (μ_{max}) of an organism can be calculated from the time and rate of wash out at a dilution rate just above D_c, the maximum dilution rate at which a steady state can be maintained.

The equation used was:

$$\mu_{max} = \frac{\ln B - \ln A}{t} + D$$

Where A = initial OD_{440}
B = OD_{440} at time t
t = time in hours

(Karagouni and Slater 1978) The maximum specific growth rate determined by this method was 0.091 h^{-1}, which corresponds

well to that calculated from batch culture (0.097 h^{-1}).

7.1.3 Enzymes induced during the growth of M2 on MSA

7.1.3.1 Methanol and formate dehydrogenase

Cell free extracts were prepared as described in section 2.10.1. Spectrophotometric tests on these extracts for the presence of methanol dehydrogenase and formate dehydrogenase proved positive. The K_m for MDH was 0.243 mM and that of FDH 3.53 mM. The V_{max} for each enzyme was 115 nmol DCPIP min^{-1} mg^{-1} protein and 47.9 μmoles NAD min^{-1} (mg dry weight)$^{-1}$ respectively. The standard assay for MDH, at pH 9.0, using a TRIS buffer showed methanol-specific and extract-dependent disappearance of DCPIP, but variation of methanol concentration did not produce results consistent enough to use in calculations of enzyme kinetics. However, if a sodium tetraborate buffer was substituted for the Tris, and KCN added to a concentration of 1 mM, then the dye-linked assay proceeded as described in the literature. Since KCN has been shown to competitively inhibit the oxidation of methanol by whole cells of M2 (section 7.5), although at a different pH (6.8), this means that MDH is inhibited to some extent in the cell-free assay used. The combination of sodium tetraborate and KCN may allow the dye-linked, methanol specific properties of the cell free extract to function with the positive or negative contribution of all or some of the following factors:

1) Inhibition of electron transfer from other enzymes, acting on substances apart from methanol (already present in

the extract itself), to PMS and DCPIP.

2) Inhibition of the MDH by TRIS.

3) Stimulation of MDH by borate, potassium or sodium.

7.1.3.2 Enzymes and carriers of the electron transport chain

Scanning spectrophotometry of cell free extracts indicated peaks in absorbance associated with cytochromes. These peaks were enhanced by the addition of a reductant, such as sodium dithionite. The peak maxima and their possible identities are shown in table 7.1.3., compared to a scan of methanol-grown cells. The membrane fraction referred to in the table was composed of cell debris and membranes spun down during the preparation of a cell free extract.

The soluble fraction of French pressed cells of M2 appeared to contain a blue pigment, with an absorbance maximum of 589.8 nm (Fig 7.1.3). There was often enough of this pigment in a cell free extract to give it a grey appearance rather than its normal red-brown colour. The pigment was only seen in MSA-grown cells, but was not detectable in every extract made. Attempts were made to enhance the amount of blue pigment in extracts by increasing the amount of copper or iron present in the trace element solution used for growth by three times. This had no effect, whether cells were grown in batch or continuous culture. The unreproducible presence of the blue pigment may have been accounted for by unknown factors associated with growth on MSA.

Membrane Associated		Soluble			
MSA-grown		MSA-grown		MeOH-grown	
Pigment	Absorption Wavelength (nm)	Pigment	Absorption Wavelength (nm)	Pigment	Absorption Wavelength (nm)
c_{553}	552.7 523.7 524.8	c_{552}	551.7 522.1 416.4	c_{551}	551.2 522.5 417.2
c_{564}	564.1 524.8	Blue	589.8		
a/a_{608}	608.2 524.8				

Table 7.1.3 Pigments in high speed fractions of strain M2. c_x = putative cytochrome of absorbance maximum x. a/a_y = putative cytochrome oxidase of absorbance maximum y. Blue refers to a light absorbing pigment of unknown function.

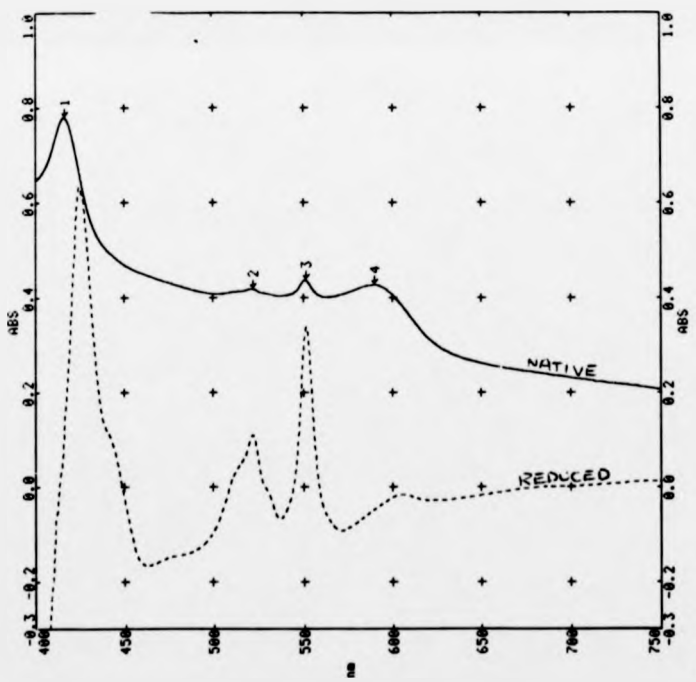


Figure 7.1.3 Scanned spectrum of a cell-free extract of MSA-grown M2. The extract was reduced using a few crystals of sodium dithionite.

The pigment itself is of approximately the same absorbance as the blue copper proteins observed by Lawton and Anthony (1985), and it could be speculated that it has similarity with either azurin or amicyanin.

The presence of enzymes associated with carbon assimilation are discussed below (section 7.2).

7.2 The assimilation of carbon from methane sulphonate

7.2.1 Introduction. Carbon assimilation in methylotrophs

Bacteria with the ability to grow on C_1 compounds are unified by common elements in their biochemistry. All the organisms studied assimilated C_1 carbon to cell carbon by variations on three pathways (figure 7.2.1). These pathways are named after the C_1 acceptor molecule and are the serine pathway, the ribulose monophosphate (RuMP) pathway and the ribulose bisphosphate (RuBP) pathway. A fourth pathway, found in methylotrophic yeasts, is a variant of the RuMP cycle, and is known as the XuMP pathway and will not be discussed in connection with bacterial C_1 metabolism. The diagrams illustrating the following pathways are found on page 151.

7.2.1.1 The serine pathway

The serine pathway was deduced from the short-term incubation of ^{14}C-labelled C_1 substrates, (Large *et al* 1961; 1962a; 1962b) with *Methylobacterium extorquens* strain AM1. The enzymes of the pathway are partly similar to those of the photoresparitory glycolate pathway (Izumi *et al* 1990b)

and essentially consist of a hydroxymethylation of glycine with formaldehyde to yield serine, a transfer of the amino group of serine to yield hydroxy pyruvate and the regeneration of glycine with the production of phosphoglycerate (see figure 7.2.1.1). This C_3 phosphorolated compound can then enter the tricarboxylic acid cycle, to furnish the bacterium with cellular constituents.

The sequence and identity of enzymes in the serine cycle seems to vary little between organisms. Some differences have been shown to exist in the method by which acetyl CoA is regenerated. The involvement of isocitrate lyase and tricarboxylic acid cycle enzymes (as shown in figure 7.2.1.1) in this process has been shown in methylotrophs such as *Pseudomonas* MA, *Pseudomonas aminovorans*, *Pseudomonas* MS and organism 5H2 (Anthony, 1982). However, *Methylobacterium extorquens* AM1 possesses no demonstrable isocitrate lyase activity, although the oxidation of acetyl CoA does result in the production of glyoxylate (Anthony, 1982). Some controversy has arisen over the validity of the only proposed route for these "icl$^-$" variant of the serine pathway, which at present remains unresolved.

7.2.1.2 The ribulose bisphosphate (RuBP) pathway

The RuBP cycle, or Benson-Calvin cycle, of autotrophic CO_2 assimilation has long been recognised in autotrophic bacteria, phototrophic bacteria, cyanobacteria and green plants. More recently, some bacteria, that could be called

autotrophic methylotrophs, have also been shown to possess the RuBP pathway. The C_1 growth substrate is oxidised to water and CO_2, providing energy in the form of NAD(P)H. The CO_2 is fixed using the enzyme RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase).

The Benson-Calvin cycle regenerates ribulose-1,5-bisphosphate, synthesizing triose phosphate from 3 CO_2. The phosphate is used in biosynthesis of cell material (see figure 7.2.1.2). This mode of metabolism is not absolute, varying from organism to organism, but ribulose bisphosphate is always regenerated.

The fixation of CO_2 can be summarised as an equation, written as follows:

$$6\ NAD(P)H + 3CO_2 + 9\ ATP \longrightarrow 3\text{-phosphoglyceraldehyde} + 6NAD(P)^+ + 9\ ADP + 8\ P_i$$

Organisms that gain carbon in this way include *Thiobacillus versutus* (Kelly and Wood, 1984) and *Paracoccus denitrificans* (van Verseveld and Stouthamer 1978).

7.2.1.3 The ribulose monophosphate pathway

The ribulose monophosphate cycle of HCHO fixation was also proposed by Quayle (1965). Since the first version of the pathway, variants have been identified, mostly in obligate methanotrophs and methanolotrophs (Figures 7.2.1.3 and 7.2.1.4.). Common features in all the variants are the synthesis of D-erythro-L-glycero-3-hexulose-6-phosphate (known as hexulose-6-phosphate for the sake of brevity) from

ribulose-5-phosphate and formaldehyde, and the isomerisation of hexulose-6-phosphate to form fructose-6-phosphate. The variation between organisms lies in the regeneration of ribulose-5-phosphate.

7.2.1.4 The concept of key enzymes

It was at first suggested that only one pathway of the three functioned in any given species of bacterium (Anthony 1975; Quayle, 1972), but work on *Pseudomonas oxalaticus* (Quayle, 1961) and work mentioned by Colby *et al.* (1979) on *Pseudomonas gazotropha* showed that one C_1 assimilation pathway may be used by an organism to enable growth on one compound, and another pathway may be used for a second compound. No convincing evidence has been presented to show the simultaneous operation of two or more pathways.

The presence of a particular C_1 assimilation pathway in a bacterium has been classically determined by the presence of one or two key enzymes, taken as being essential to the functioning of the pathways. Hydroxypyruvate reductase (HPR, see figure 7.2.1.1) has been taken as an indicator of the presence of the serine pathway, hexulose phosphate synthase (HPS, see figure 7.2.1.3 and 7.2.1.4) an indicator of the RuMP pathway and both RUBISCO and phosphoribulokinase an indicator of a RuBP pathway. However, the presence of an enzyme is no absolute indicator of the method of carbon assimilation.

The methanotroph *Methylococcus capsulatus* (Bath) has RUBISCO and phosphoribulokinase, despite having all the enzymes of

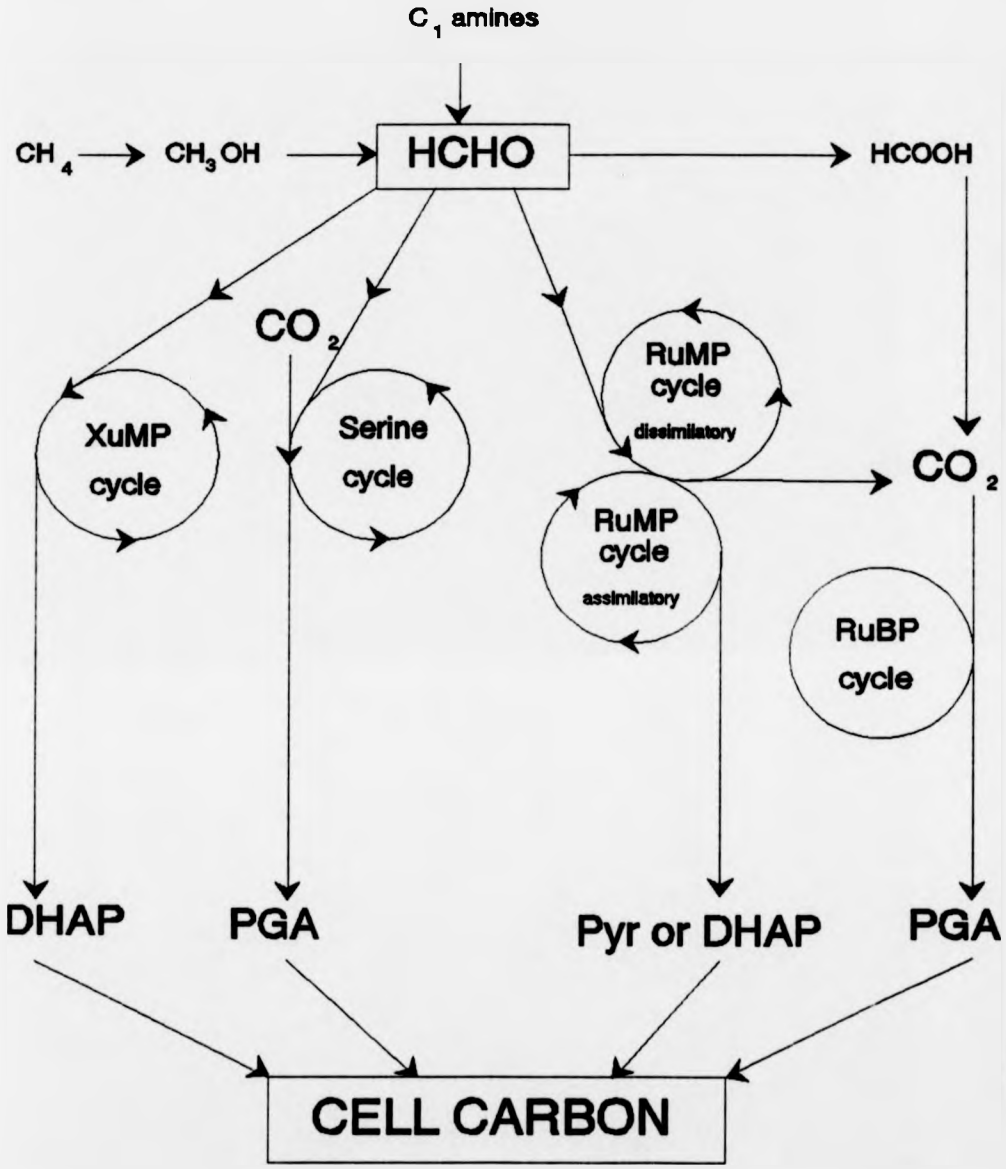


Figure 7.2.1 Assimilatory and dissimilatory pathways in methylotrophs (After Zatman, 1980)

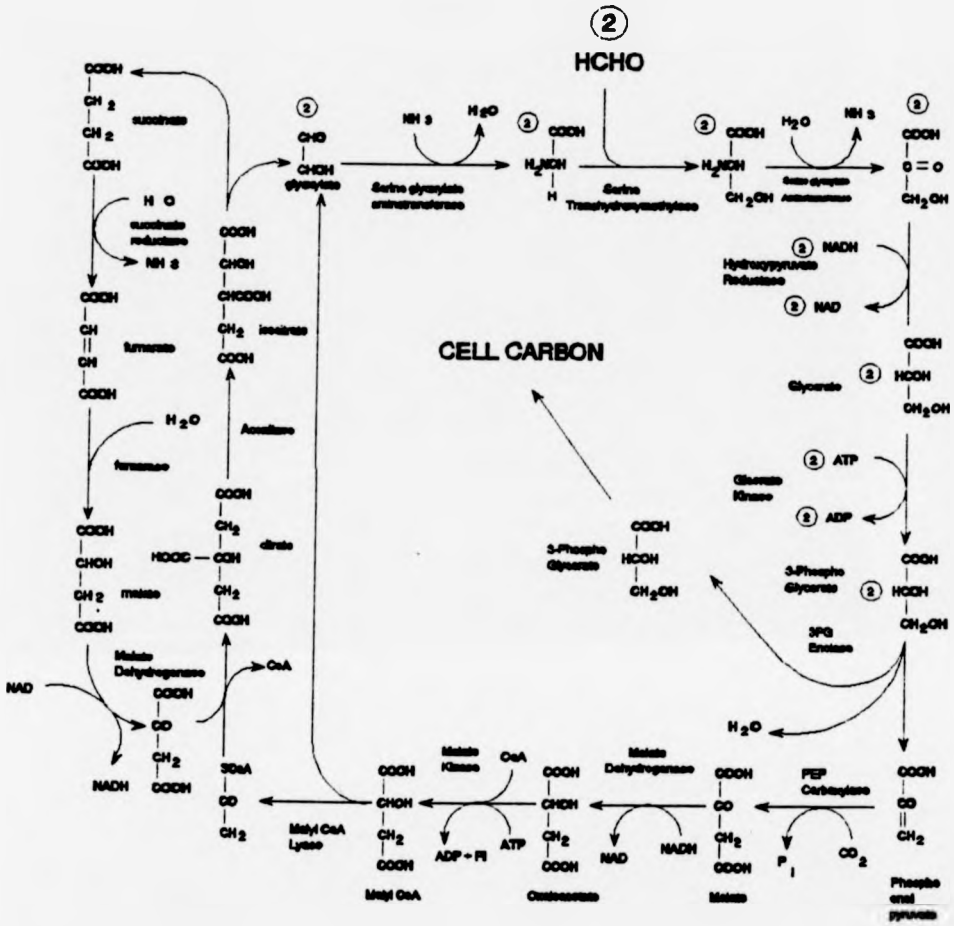


Figure 7.2.1.1 The serine pathway for the assimilation of carbon during methylotrophic growth.

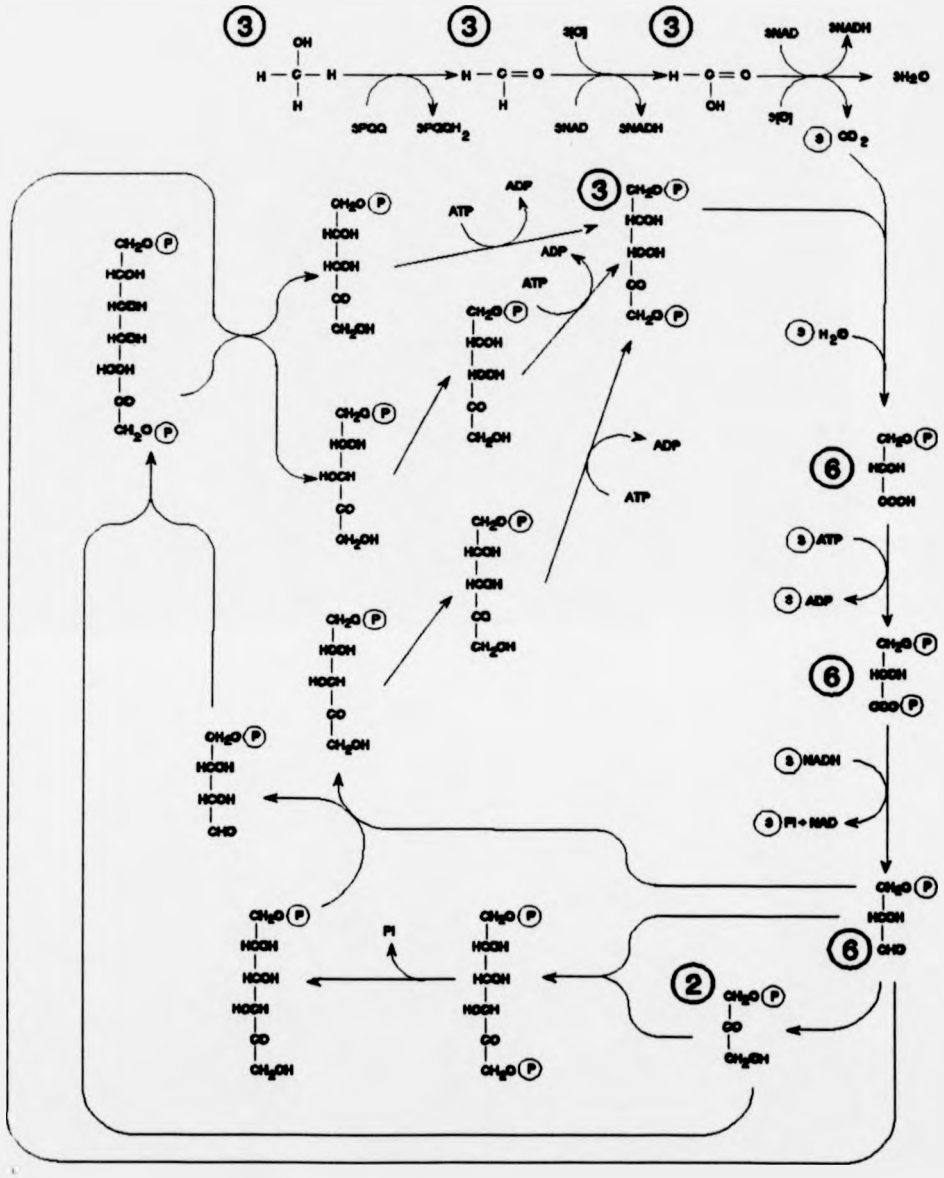


Figure 7.2.1.2 The assimilation of carbon by methylotrophic autotrophs. Encircled numbers indicate the stoichiometry of the transformations. PQQ = pyrroloquinoline quinone. $PQQH_2$ = reduced pyrroloquinoline quinone

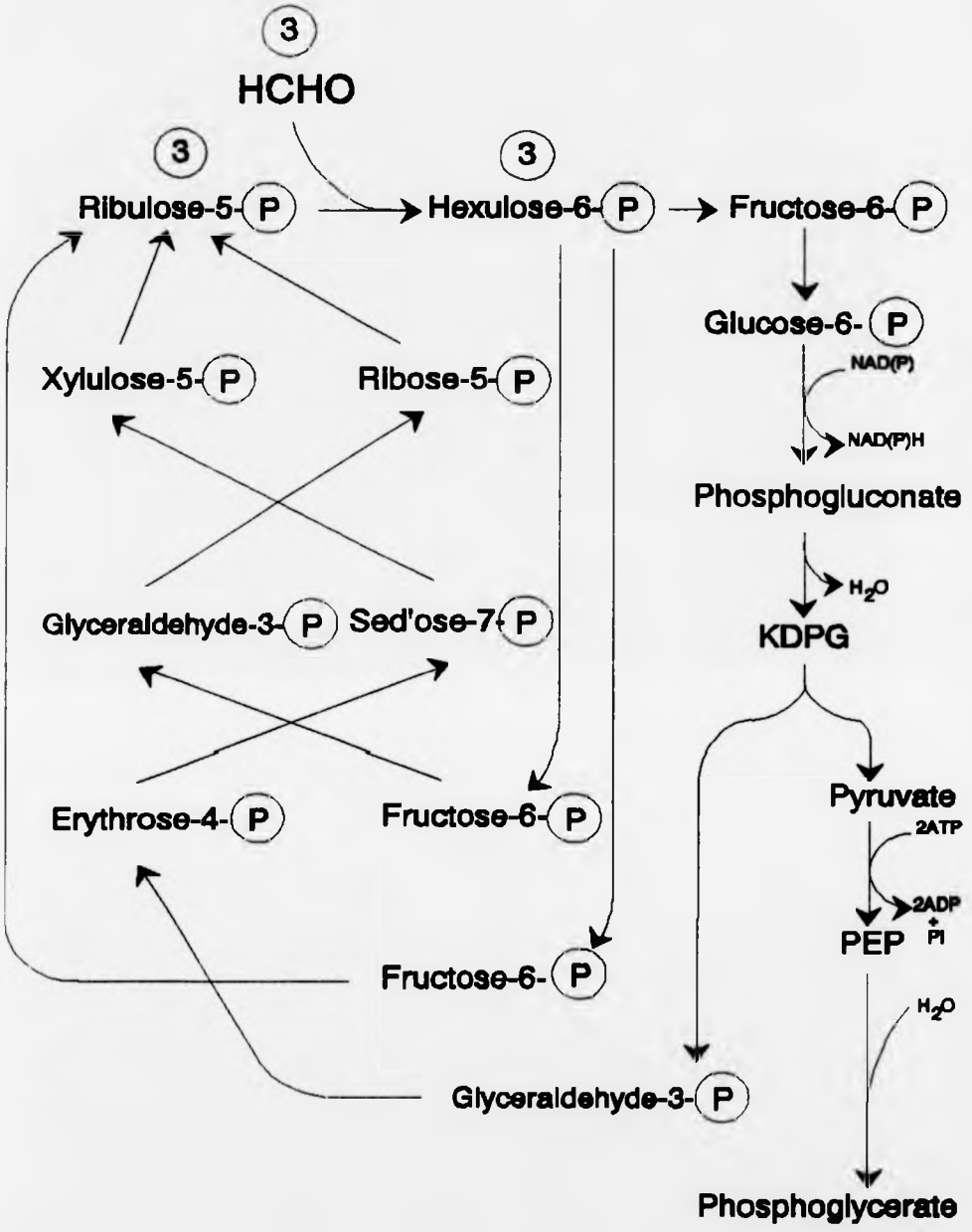


Figure 7.2.1.3 The KDPG variant of the ribulose monophosphate pathway for the assimilation of carbon during methylotrophic growth.

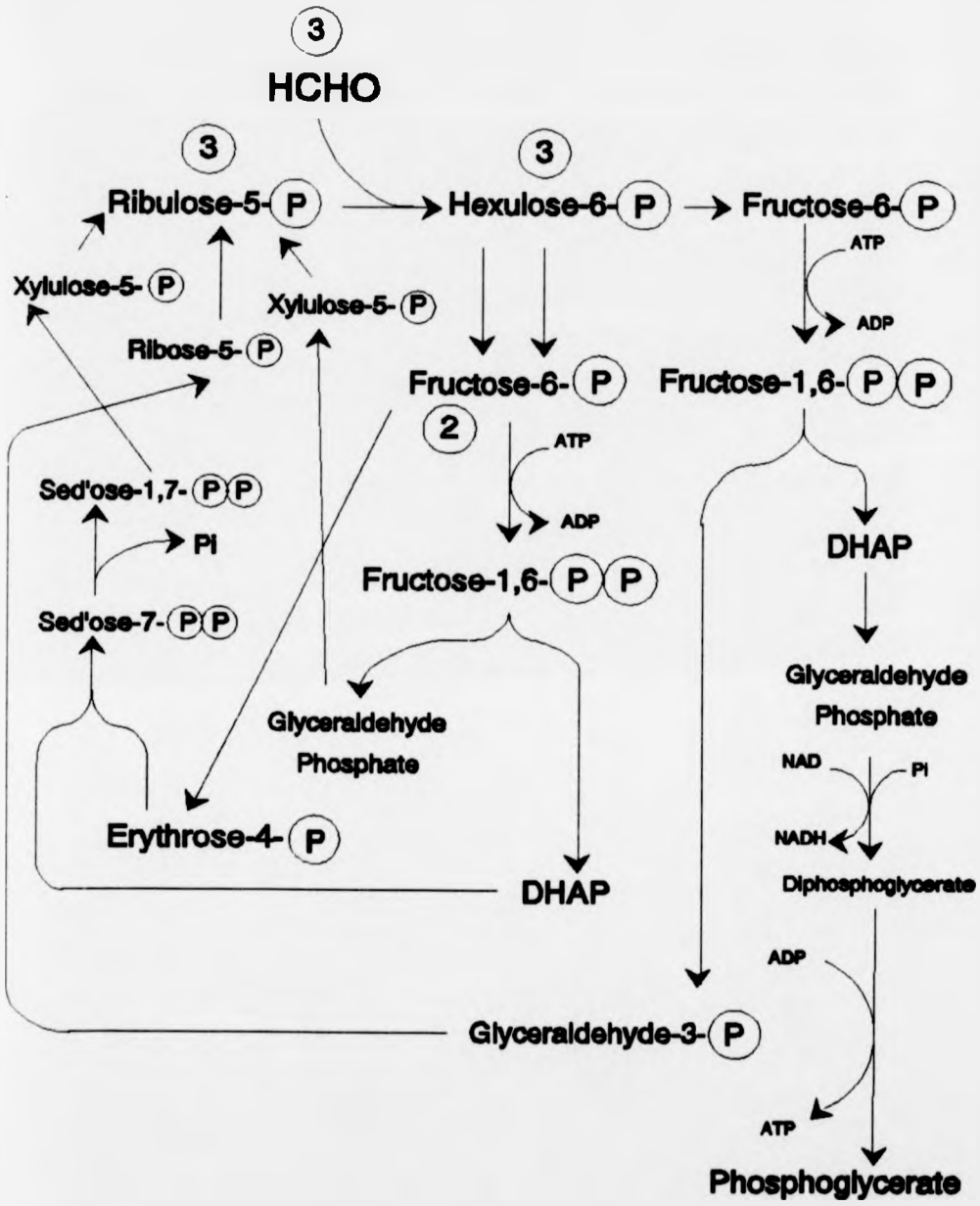


Figure 7.2.1.4 The fructose bisphosphate variant of the ribulose monophosphate pathway for the assimilation of carbon during methylotrophic growth.

the RuMP cycle (Taylor, 1977). Conversely, *Paracoccus denitrificans*, uses an RuBP pathway for growth on compounds such as methanol, but has constitutive HPR activity of unknown function (Bamforth and Quayle, 1977), which has lead to the suggestion that other enzymes exist capable of reducing hydroxypyruvate, but of another primary function. Despite these anomalies, assays of single key enzymes can still indicate which pathway is used, as long as the relative activities of the enzymes are disparate.

7.2.2 Conversion of formaldehyde to cell carbon by strain M2

The presence of HPR and HPS were assayed in cell-free extracts of MSA-grown M2. It was found that hexulose phosphate synthase was absent, but was easily detectable in *Methylococcus capsulatus* (Bath), assayed as a control. Strain M2 cannot, therefore, use the RuMP cycle for carbon assimilation (Colby *et al.*, 1979). Hydroxypyruvate reductase was detected: extracts showed hydroxypyruvate-dependent NADPH oxidation activities of 589 nmol NADPH per minute per mg protein in unoptimised conditions, but no activity with NADH. This would suggest that in M2 the serine pathway is used for the assimilation of carbon during growth on MSA.

7.2.3 Conversion of carbon dioxide to cell carbon by strain M2

The amount of $^{14}CO_2$ incorporated into cell carbon was measured during growth of M2 on MSA. The amounts of ^{14}C remaining in the medium and in the cell were compared with the biomass of the culture (Figure 7.2.3.1). Approximately

20% of the cell carbon was derived from CO_2, a figure more consistent with a bacterium possessing a serine pathway than a RuBP pathway. In the case of an RuBP organism, CO_2 fixed may account for virtually all of the cell carbon. An organism with the serine pathway incorporates CO_2 during the regeneration of serine, when phospho-enol pyruvate carboxylase catalyses the formation of oxaloacetate. Nothing is known of the central routes of anabolism and catabolism in M2, but presumably CO_2 fixation could have occurred within these pathways (Eg production of oxaloacetate from pyruvate by pyruvate carboxylase in the pentose phosphate pathway).

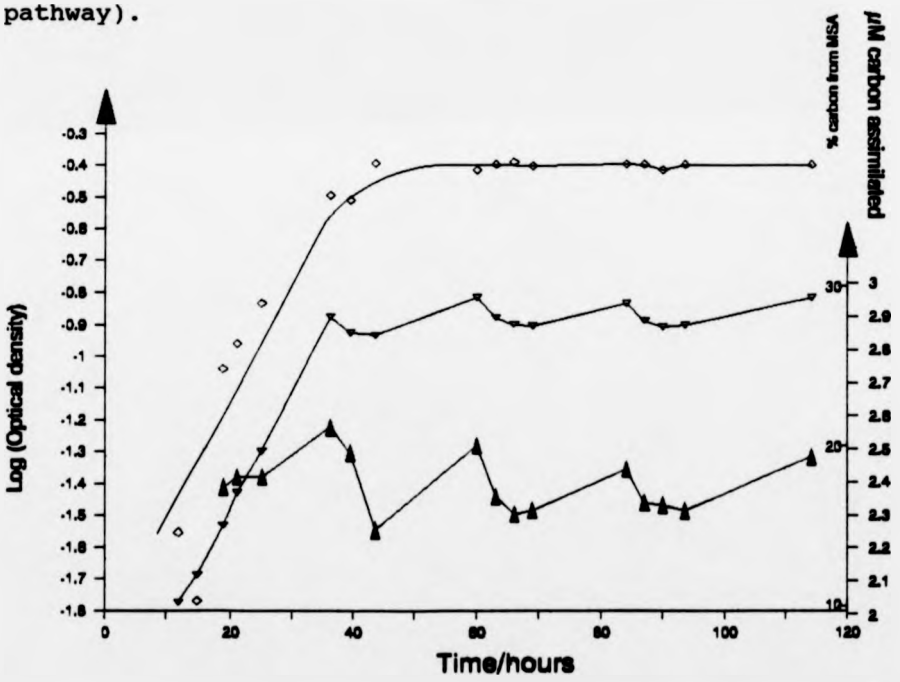
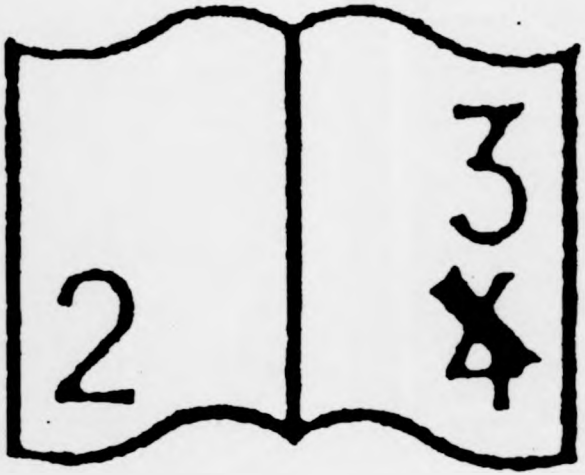


Figure 7.2.3.1 Growth and assimilation of carbon from CO_2 by M2 growing on 10 mM MSA. ◇ = OD_{440}, ▼ = µmoles carbon assimilated, ▲ = carbon assimilated expressed as a percentage of all the cell carbon.

PAGINATION ERROR



191

To check that CO_2 observed uptake did not represent an autotrophic mode of growth, the RUBISCO assay was performed three times on whole cells with varying concentrations of detergent. Work by M. Maclean (University of Warwick) showed that enhanced detection of RUBISCO was enabled under these conditions. The lowest concentration of detergent used seemed to suggest that CO_2 fixation was occurring in MSA cells, but this result could not be repeated. Similarly, RUBISCO could not be detected reproducibly in cell-free extracts. Since RUBISCO could not be definitively shown to be present, less direct methods were employed.

The presence of RUBISCO was detected non-quantitatively in cell-free extracts of M2 by immuno-blotting a polyacrylamide gel, indicating that RUBISCO was indeed present. This result is discussed further, with reference to the growth of M2 on formate, in section 8.2.1.

7.3 Possible routes of assimilation of MSA

7.3.1 Introduction

Assays of the key enzymes of methylotrophic carbon fixation have established that M2 grows on MSA using the serine pathway to assimilate carbon. Thus it was postulated that formaldehyde was an intermediate in the metabolism of MSA, as this is the entry point of C_1 carbon units to the serine pathway in every other organism cited in the literature. However, this lead to

little information with regard to the primary reactions needed to oxidise MSA to the level of formaldehyde. Several likely schemes were prepared and are illustrated in figure 7.3.1.1.

In summary, these four selected schemes are the generation of methane (scheme 1), an oxidation to methanol (scheme 2), an oxidation directly to formaldehyde (scheme 3) and oxidation of a carrier molecule side chain (scheme 4).

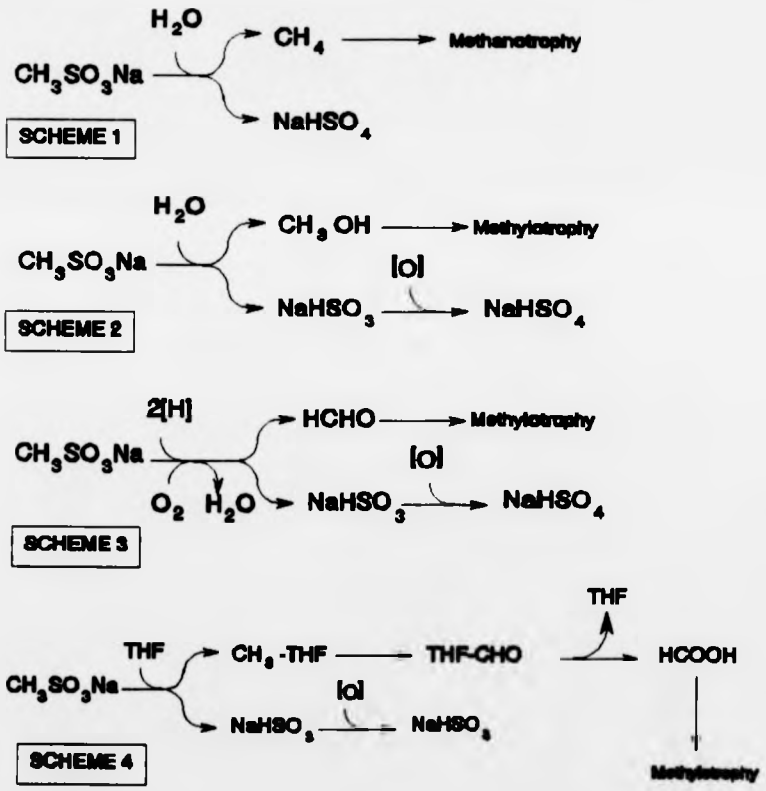


Figure 7.3.1.1 Possible mechanisms for the degradation of MSA. THF denotes a "carrier" molecule, similar in function to tetrahydrofolate in Pseudomonas MS.

7.3.2 Methane as a primary product in the degradation of MSA (Scheme 1)

A close structural analogue of MSA, methane phosphonate (MPA), is used as a phosphorus source by several organisms including *Escherechia coli* and *Pseudomonas testosteroni* (See section 6.1). The C_1 compound is not assimilated in this heterotroph, as methane is released but the organisms can derive phosphate from the process. A similar process could be suggested for M2 growing on MSA. Methane, rather than being released could act as a source of cell carbon, its oxidation providing energy. Sulphate would be the other product of the initial cleavage.

The main evidence against the cleavage of methane from MSA playing any part in the oxidation of MSA in M2 was three-fold. Methane was not a growth substrate for M2 (see table 6.3.3.1), nor an oxidation substrate of MSA-grown cells and it could not be detected at any time during the organism's growth cycle by GC. The generation of methane as a degradative route for MSA will not be discussed further in this thesis.

7.3.3 Methanol as a primary product in the degradation of MSA (Scheme 2)

The methane sulphonate molecule has its weakest bond connecting the carbon and sulphur atoms. If this bond is the site of the first enzymatic cleavage and the products would be a methyl group and a sulphonate group, attached

to some other molecule or atom. The subsequent steps in the oxidation of these groups would lead to methanol and sulphite.

7.3.4 Formaldehyde as a primary product in the degradation of MSA (Scheme 3)

A monooxygenic enzyme catalysed attack on the methane sulphonate molecule could lead to the direct formation of formaldehyde (figure 7.3.4).

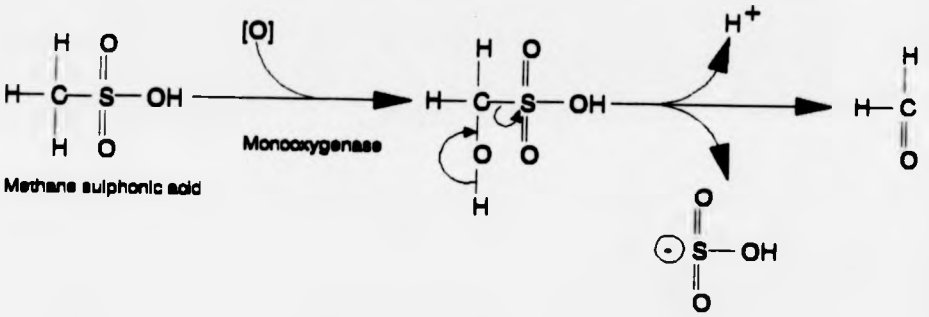


Figure 7.3.4 The metabolism of MSA to yield formaldehyde as a primary product.

Formaldehyde might then be subjected to further oxidation to formate, then CO_2 and water, or be used for assimilation into cell carbon. This pathway uses the same amount of oxygen to effect complete mineralisation of MSA as scheme 2, but may conceivably not result in the production or use of the same cofactors. The model that results in the production of methanol is conceptually

similar to the methanol and methane oxidation pathways found in many methylotrophs and methanotrophs respectively, the oxidation from the level of methanol producing resulting in the formation of reducing power at each stage of the mineralisation. The amount of reducing power generated from the direct oxidation of MSA to formaldehyde may differ from the conventional pathway, but that from the mineralisation of formaldehyde would be the same.

7.3.5 Oxidation of a carrier molecule side-chain (Scheme 4)

Methylotrophs such as *Pseudomonas* MS (Kung and Wagner, 1970), accomplish the oxidation of compounds containing methyl groups, such as trimethyl sulphonium chloride, by transferring the group to a carrier molecule. In the case of *Pseudomonas* MS this molecule is tetrahydrofolate. The methyl group is then oxidised as a side chain of the larger carrier molecule, and released from it as formate, which can then be oxidised to CO_2 and water. The carrier molecule with an aldehyde side chain can also be fed directly into, for example, the serine pathway of carbon assimilation as $N^{5,10}$methlyenetetrahydrofolate. Such a system could allow the oxidation of the methyl group of MSA, after it had been split from the sulphonate moiety. Equally, a carrier molecule could be used to accept the whole molecule:

$$\text{Carrier} + CH_3SO_3Na \longrightarrow \text{Carrier-}CH_2SO_3Na$$

This would allow the oxidation of the side chain to proceed via either of the pathways outlined in sections 7.3.3 and 7.3.4. Thus the questions surrounding the route of oxidation of MSA can be applied to hypothetical pathways involving free or bound methyl and aldehyde groups, without elementary identification of the identity of the putative carrier molecule.

7.3.6 Comments

The work on M2 has, for the major part, been directed towards elucidating the pathway of MSA oxidation, whether by scheme 2 or scheme 3. Since neither compound could be detected in media during growth of M2 on MSA, two approaches have been taken to induce the accumulation of an identifiable intermediate. These have been to inhibit the methanol-oxidising activity of MSA-grown cells and to observe the oxidation of MSA analogues by MSA-grown cells.

7.4 The oxidation of methane sulphonate

7.4.1 Introduction

The complete oxidation of MSA requires the involvement of two molecules of oxygen:

$$CH_3SO_3Na + 2O_2 \longrightarrow CO_2 + H_2O + NaHSO_4$$

If an oxygenase is involved in the initial step of the pathway, then an additional 0.5 mol oxygen is required:

$$CH_3SO_3Na + 2\tfrac{1}{2}O_2 + 2[H] \longrightarrow CO_2 + 2H_2O + NaHSO_4$$

Stoichiometry is the amount of one substrate used compared to the amount of another substrate, in a given reaction. This parameter can be applied to the metabolism of MSA: the stoichiometry of the first reaction with regard to oxygen is 2, and in the second it is 2½. The consumption of oxygen in the presence of a substrate can be measured using the oxygen electrode, and is calibrated by a method based on a supposition that aerated distilled water contains 245 nmole of oxygen ml^{-1}. The calibrated electrode can yield the amounts and rates of use of oxygen in the presence of substrates. Modes of oxidation can be deduced from stoichiometries, based on a knowledge of the chemistry of the substrate.

7.4.2 Substrate specificity of MSA-grown cells

The methylotrophic strain M2, when grown on MSA, oxidised few compounds (see table 7.4.1).

Potential substrate analogues (ethane sulphonate, methane phosphonate, monomethyl sulphate) were not oxidised, which could lead to the hypothesis that the enzyme(s) involved in the first stages of MSA metabolism (including any involved with transport across the membrane of the cell), are specific.

The absence of any oxygen consumption in the presence of ethylene suggests that the organism does not have the capacity for epoxidation. This result was repeated using cell-free extracts,

Compound	Stoichiometry	K_m mM	V_{max} nmol O_2/min/mg dry wt
MSA•	1.5	0.02	229
MeOH•	1.0	0.01	355
HCHO•	0.5	0.08	198
HCOOH•	*	*	*
NH_2MSA•	0	NA	NA
Formamide	0	NA	NA
MMSA	0.5	NT	NT
ESA	0	NA	NA
MPA	0	NA	NA
MMS	0	NA	NA
$MeNO_2$•	0	NA	NA
MMA•	0	NA	NA
DMA•	0	NA	NA
TMA•	0	NA	NA
TMS•	0	NA	NA
Pyruvate	0	NA	NA
Methane	0	NA	NA
Ethanol	0	NA	NA
Glucose•	0	NA	NA
Acetate•	0	NA	NA
Ethylene	0	NA	NA
Thiosulphate	0	NA	NA
Benzene sulphonate	0	NA	NA
Sulphite	0	NA	NA

Table 7.4.1. Oxidation substrates of the methylotroph M2 grown on MSA. NA = not applicable, NT = not tested, * = No detectable oxidation unless concentration of formate in the oxygen electrode was more than 16 mM. "•" denotes a growth substrate. All solutions of compounds were made up in M2 buffer and the pH adjusted to 7.0 before use. NH_2MSA = aminomethane sulphonate, MMSA = methyl methane sulphonate, ESA = ethane sulphonate, MPA = methyl phosphonate, MMS = monomethyl sulphate, DMA = dimethylamine, TMA = trimethylamine, TMS = trimethyl sulphonium chloride.

but due to the apparent lability of MSA-dependent oxygen consumption (see below), significance cannot be attributed to this result. The epoxidation of a double bond has been used as evidence for the presence of a monooxygenase (Colby *et al*, 1977), so in this respect the oxidation of M2 may differ from the NADH- and oxygen -dependent cleavage of C_4-C_{12} *n*-alkane-1-sulphonates demonstrated in *Pseudomonas* (Thysse and Wanders, 1972; 1974).

7.4.3 MSA oxidation by MSA-grown cells

The oxidation of MSA itself could not be performed with cells taken straight from an MSA-limited chemostat or batch culture. Cells such as these had an endogenous rate of respiration high enough to mask any increase due to the addition of other compounds. The cells lost this endogenous respiration after washing three times with M2 buffer at room temperature. Cells suspended in buffer lost their ability to oxidise MSA after an hour's storage on ice, but retained that to oxidise methanol and formaldehyde. Washed cells frozen as a slurry with liquid nitrogen and stored at -70 °C also lost the ability to oxidise MSA, as did washed cells frozen at -20 °C, again retaining methanol and formaldehyde oxidative capabilities.

The apparent loss of the MSA "oxidase" was reflected in the properties of cell free extracts as well. An increase in oxygen consumption (measured in the oxygen electrode) could not be stimulated by the addition of MSA to an extract, whether in the presence or absence of membranes, or with the addition of $NA(P)D^+$, NAD(P)H or ATP. No disappearance of these cofactors could be detected spectrophotometrically, above an endogenous, MSA-independent rate. The labile nature of the MSA "oxidase" in whole cells suggests that the lack of oxidation of MSA in cell free extracts is due to the relatively harsh preparative methods used, rather than the absence of a particular factor.

7.4.4 The biphasic oxidation of MSA by whole cells

When MSA is introduced into an oxygen electrode containing a suspension of M2, the expected result would be that for every mole of MSA present, the electrode would 2 two moles of oxygen (or 2½ if an oxygenase is involved) disappearing, this disappearance having taken place at a constant rate. This would be consistent with the complete oxidation of MSA to water and CO_2. The theoretical model did not match the results obtained. When the number of moles of MSA did not exceed half the amount of oxygen present in the electrode (i.e. ~122 nmoles), the stoichiometry of the reaction revealed that the oxidation of one mole of MSA resulted in the consumption of only 1.5 moles of oxygen. Rather than complete oxidation, this suggests that whole cells grown on MSA catalyse the following reaction:

$$CH_3SO_3H + 1\tfrac{1}{2}O_2 \longrightarrow HCOOH + H_2SO_4$$

If an oxygenase has a role, then whole cells could be said to be catalysing the reaction:

$$CH_3SO_3H + 1\tfrac{1}{2}O_2 + 2[H] \longrightarrow HCHO + H_2SO_4 + H_2O$$

This result was repeated if the substrate oxidised was methanol or formaldehyde, the stoichiometry being 0.5 below that expected (see table 7.4.2). Formate did not stimulate the consumption of oxygen, except at concentrations above 16 mM.

Compound	Expected stoichiometry	Stoichiometry
MSA	2.0 (2.5)	1.5
Methanol	1.5	1.0
HCHO	1.0	0.5
HCOOH	0.5	*

Table 7.4.2 Stoichiometric results with regard to oxygen obtained from suspensions of whole cells of M2 incubated in the oxygen electrode with several substrates. Figures in brackets refer to the stoichiometry of the named reaction if a monooxygenase is involved. * = not determined (see text).

Although formate cannot be detected in the supernatant of a sample from a chemostat with MSA as sole carbon source, variable concentrations can be detected throughout the growth of batch cultures (see figure 7.4.4.1).

On closer examination of the rate at which MSA is oxidised in the oxygen electrode, a prolonged incubation using a slower chart speed revealed that the oxidation of MSA is biphasic. The faster part of the oxidation was observed previously, but the much slower oxidation ignored and taken to be a return to endogenous levels of oxidation, as described earlier in this section. A sample trace, compressed horizontally, is shown in figure 7.4.3.2.

The presence of formate in culture supernatants and the biphasic oxidation of MSA would imply that the sulphonate was partially oxidised within the cell to formate. The activity of formate dehydrogenase in cell free extracts of MSA-grown and formate-grown cells were similar (see section 8.4), suggesting no reason why formate oxidation should be slower.

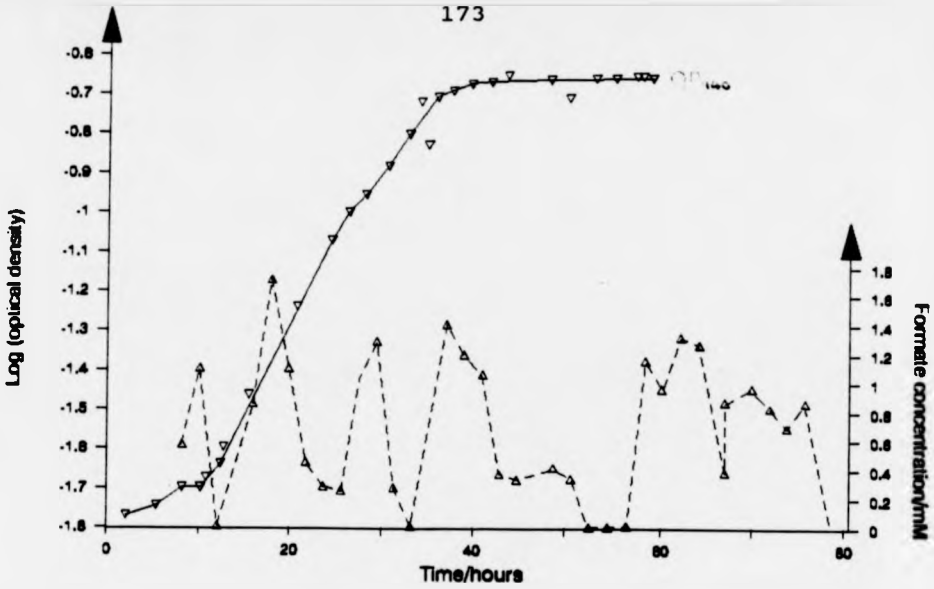


Figure 7.4.4.1 Formate concentration in the medium during growth of M2 on MSA.

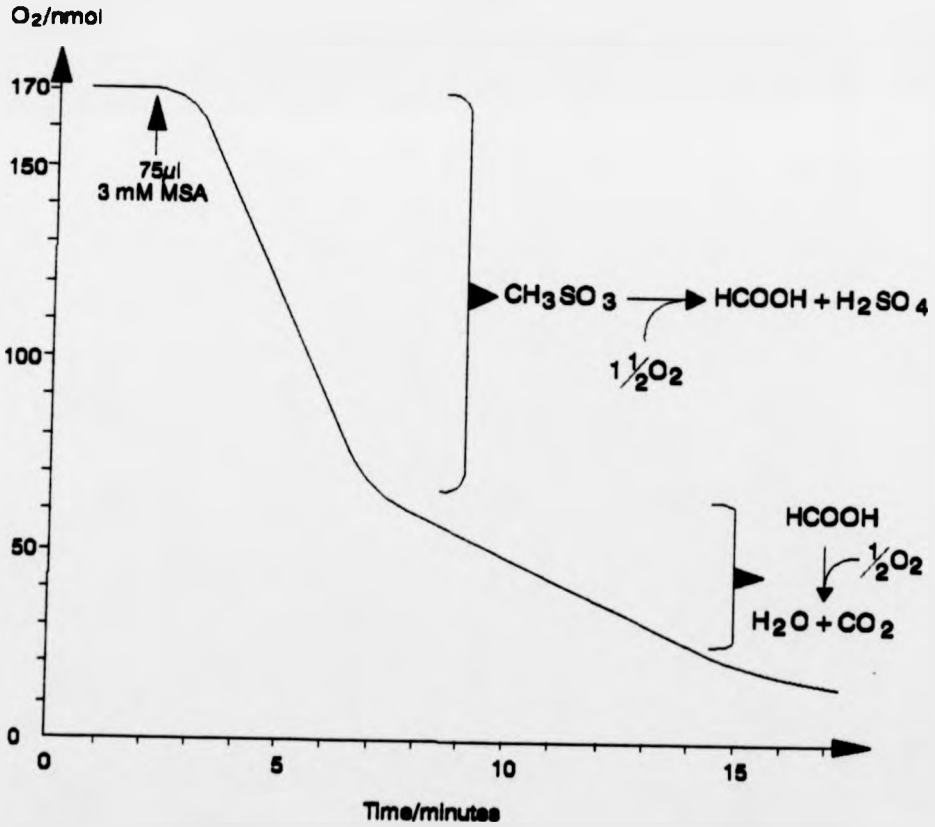


Figure 7.4.3.2 The biphasic nature of the oxygen consumption stimulated by MSA.

Formate oxidation has been associated to the maintainence of NADH balance within the cell, as formate dehydrogenase is an NAD-linked enzyme that in non-autotrophic organisms is non-essential fro growth. Thus the regulation of this enzyme will regulate NADH levels within the cell without affecting the capacity for growth. Given that the FDH of MSA-grown M2 is active, yet whole cells will not oxidise formate, one could propose a scheme whereby formate is transported across the membrane of the cell and import switched off. In an actively growing cell this would provide NADH limitation without direct repression of FDH. However, this scheme is probably over-complicated for essentially a simple regulation. Anthony (1982) maintains that many methylotrophs are NADH-limited rather than carbon-limited, so it is difficult to see why M2 should need to repress FDH or formate oxidation, and thus a source of NADH.

In conclusion, the effects seen with regard to formate oxidation are most likely to be an artifact of the methods of preparation of whole cells. It may have been that some ion in the M2 buffer used in the experiments inhibited formate import, and so no formate oxidation could be seen. This still leads to no satisfactory explanation for the presence of formate in the medium of batch cultures, or for the biphasic oxidation of MSA, but these may have to be regarded as peculiarities of M2's oxidation capabilities until more detailed studies can be performed.

7.4.5 The fate of sulphite ions cleaved from MSA

The sulphite generated from the oxidation of MSA, a possible product of the first cleavage, was oxidised to sulphate by chemical means only. When fresh sodium sulphite (made up in water degassed with oxygen-free nitrogen) was supplied to MSA-grown cells contained in an oxygen electrode, no additional oxidation could be detected above the rate created by the action of oxygen from the air.

7.5 The inhibition of methanol oxidation

MSA-grown cells contain methanol dehydrogenase (MDH, see section 7.2.4), but this in itself is not an indicator that the metabolism of MSA includes methanol as an intermediate. The strain M2 contained MDH irrespective of what C_1-compound the organism was grown on (see section 8.4). The presence of MDH in MSA-grown cells could have led to the theory that MSA was an alternative substrate for MDH, but this is refuted by the inability of methanol-grown cells to oxidise MSA (section 8.2).

If the assimilation of carbon from MSA proceeded via scheme 3, with a primary product of formaldehyde, then it should be possible for the cell to grow on, or fully oxidise MSA in the presence of an inhibitor of methanol oxidation. If, however, methanol oxidation is required, then no growth will occur in the presence of such an inhibitor, and the stoichiometry of an MSA oxidation under these conditions will not rise above 0.5. Several suitable inhibitors were identified from the literature. The ideal properties of an

inhibitor were that the inhibitor should stop or slow down the action of methanol oxidation and that the inhibitor should have no effect on the oxidation of MSA.

7.5.1 Phosphate concentration.

The concentration of phosphate has been shown to inhibit dehydrogenases in whole cells (A. Midhir, unpublished results). MinE was prepared containing varying concentrations of mono- and dipotassium hydrogen phosphate. The ratio of mono- to dipotassium hydrogen phosphate was kept constant so that the pH of the medium remained at 6.8. The flasks were subasealed after autoclaving, substrate added and inoculated with a thick suspension of MSA-grown cells before incubating in a shaking water bath at 30 °C. Samples of culture supernatants were taken over a period of eight hours, during which time no methanol could be detected by GC.

7.5.2 2,4-Dinitrophenol (DNP), Ethylene diamine tetraacetic acid (EDTA) and p-Nitrophenol hydrazine

Anthony and Zatman (1964) prepared cell-free extracts of bacterium M27 and demonstrated inhibition of MDH using DNP, EDTA and *p*-nitrophenol hydrazine. When saturated aqueous DNP was added to cells oxidising MSA or MeOH, the substituted phenolic had no effect. The oxidation of MSA and MeOH by whole M2 cells were not affected by 5 mM EDTA either, nor did *p*-nitrophenol hydrazine have any effect on the capacity of whole M2 cells to oxidise MSA or MeOH. Pre-incubation of the cells with these inhibitors had no effect, on the

oxidation of MSA or MeOH.

7.5.3 Potassium cyanide (KCN)

Potassium cyanide is known to be a potent metabolic poison, acting on the terminal electron oxidase of many electron transport systems. If MSA oxidation was coupled to such a system, then KCN might have acted as an inhibitor. Although it proved to inhibit the oxidation of MeOH by whole MSA-grown M2 cells by 90% when at a concentration of 2 mM, it was also an efficient inhibitor of MSA, reducing the rate of oxidation by 50% at a concentration of 0.5 mM. The oxidation of MSA would stop completely if the concentration of KCN was increased to 1 mM. Thus although KCN inhibits methanol oxidation, it can provide no information as to the product(s) of MSA oxidation.

7.5.4 Cyclopropanol

Duine and co-workers (Dijkstra *et al*, 1984) have shown that cyclopropanol is an efficient inhibitor of methanol oxidation in many organisms, whether in whole cells or cell-free extracts. This compound could have been an ideal inhibitor with which to establish the involvement of MDH in the oxidation of MSA. However, it is not available commercially, and time needed to complete the synthesis and purification of the compound was prohibitive.

7.5.5 Discussion

The failure of any of the compounds tested to inhibit methanol oxidation (summarised in table 7.5.7) are more an indication of

the difficulties involved in working with whole cells rather than any unique properties of the MDH of strain M2. There is no doubt as to the presence of MDH. Apart from the oxidation studies and enzyme assays confirming its presence, polymerase chain reaction work by V. McGowan (University of Warwick, unpublished results) using DNA isolated from M2, have isolated nucleic acid sequences in common with the MDH of *Methylococcus capsulatus* (Bath), *Methylobacterium* AM1 and many other methano- and methylotrophs.

Growth Substrate	Oxidation Substrate	Inhibitor	Effect
MSA	MSA	2,4 DNPH 0.167 mM	None
MSA	MeOH	2,4 DNPH 0.167 mM	None
MSA	MSA	EDTA 5 mM	None
MSA	MeOH	EDTA 5 mM	None
MSA	MSA	*p*-NPH	None
MSA	MeOH	*p*-NPH	None
MSA	MSA	KCN 0.5 mM	50% inhibition
MSA	MSA	KCN 1 mM	100% inhibition
MSA	MeOH	KCN 2 mM	90% inhibition

Table 7.5.7 Inhibition of the oxidation of MSA and MeOH by MSA-grown cells of strain M2. "50% inhibition" signifies a 50% reduction in the rate of oxidation of a compound. 2,4-DNP = 2,4-dinitrophenol, EDTA = ethylene diamine tetraacetic acid, KCN = potassium cyanide, p-NPH = p-nitrophenol hydrazine.

7.6 Methane sulphonate structural analogues

7.6.1 Introduction

A structural analogue of a substrate may be acted upon in much the same way as the original substrate, but the products of that reaction may not be suitable for further metabolism. Analogues of MSA, with extra groups bound to the carbon (aminomethane sulphonate and ethane sulphonate) or sulphur atom (methylmethane sulphonate), were used as possible growth and oxidation substrates.

7.6.2 Aminomethane sulphonate (NH_2MSA)

The amine derivative of MSA was selected as the molecule most likely to elucidate the mode of mineralisation of MSA.

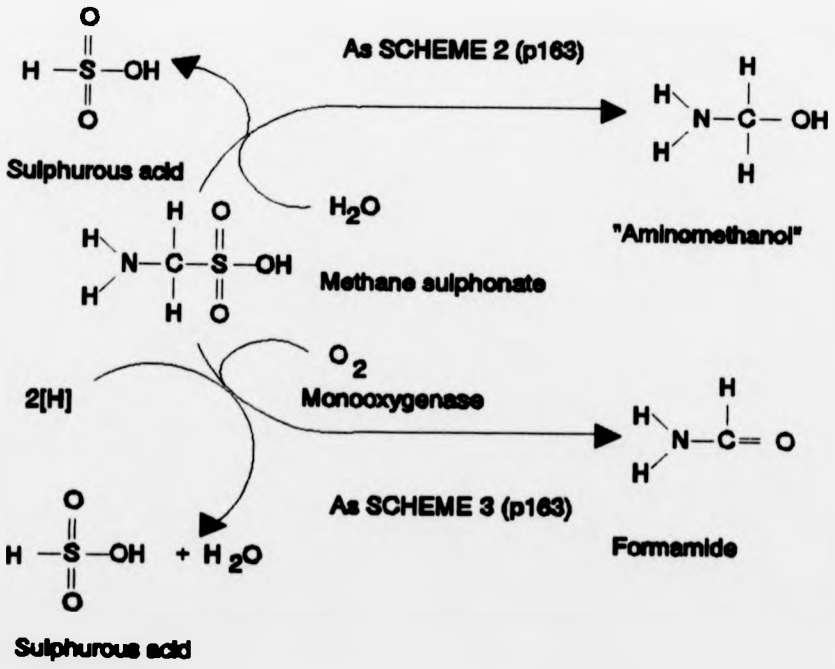


Figure 7.6.2.1 Theoretical enzymatic pathways for the oxidation of NH_2MSA.

The position of the amine group meant that the theoretical degradation of NH_2MSA by M2 could be followed by the presence or absence of easily detectable intermediates. These intermediates are shown in figure 7.6.2.1.

Two possibilities could have arisen. If the primary attack on MSA resulted in the production of methanol, when NH_2MSA was oxidised by the same system of enzymes, the structural equivalent of methanol, aminomethanol would be the intermediate. However, this compound is unstable and could only exist transiently, or would not be formed. The latter was the more likely, as the energy involved in attaching amino and hydroxy groups to a single carbon atom are prohibitive (S. Borneman, University of Warwick, Department of Chemistry, personal communication). Thus if the MSA metabolism pathway in scheme 2 (suggested in figure 7.3.1.1) was in operation and aminomethanol was an energetically unfavourable intermediate, NH_2MSA would not be oxidised by M2 cells or act as a growth substrate for them.

The other possibility was if the primary attack on MSA resulted in the direct formation of formaldehyde. Structurally, the equivalent of formaldehyde is formamide, a compound easily detectable by GC. Since this is a toxic compound it was hoped that if the MSA metabolism pathway in scheme 3 (suggested in figure 7.3.1.1) existed, then NH_2MSA would effectively act as a suicide substrate.

It was found that M2 could neither grow on or oxidise formamide, so if a thick cell suspension was supplied with

NH_2MSA, either product (formamide, indicating scheme 3) would have accumulated or nothing would have happened (indicating scheme 2), according to the hypothesis above. However, when cells were incubated at 30 °C with 15 mM NH_2MSA in MinE, no formamide in supernatants was detected by GC or NMR. One effect was noted. The optical density of the cells had risen slightly. Inoculation of fresh M2 into MinE containing 5 and 15 mM NH_2MSA confirmed that M2 can grow on NH_2MSA, despite the theory that it would be a suicide substrate.

Polyacrylamide gel electrophoresis showed similarity in the molecular weights of the proteins present in MMA-grown cells and NH_2MSA-grown cells. This suggested that NH_2MSA was deaminated as a primary step, then presumably the resulting MSA was fed into the normal, still unidentified, pathway.

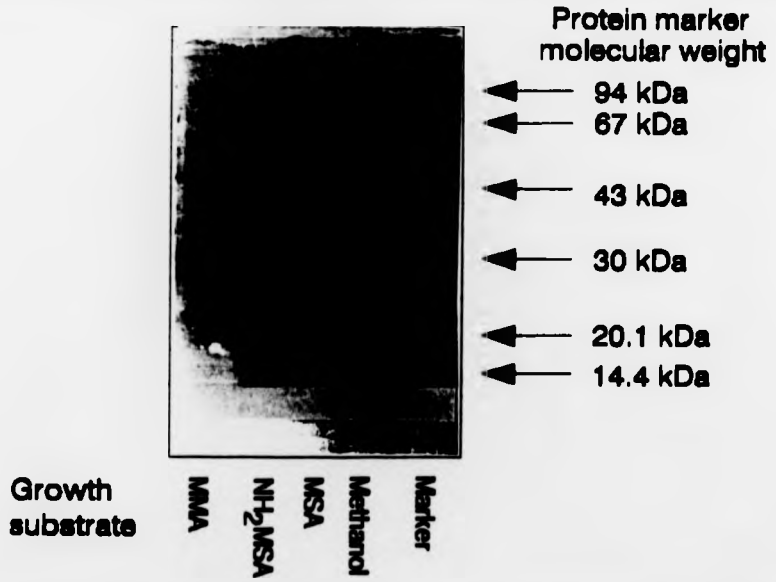


Figure 7.6.2.2 Poly acrylamide gel electrophoresis to display the protein profiles of M2 when grown on several substrates.

7.6.3 Methyl methane sulphonate (MMSA)

The sulphonate equivalent of a methyl ester, methyl methane sulphonate, is a carcinogen sometimes used to create multiple deletions in DNA in order to isolate mutants of a bacterium. Thus the observation that MMSA is not a growth substrate for M2 at concentrations of 5 or 15 mM is not unsurprising. Nonetheless, MMSA might have had some action on the enzyme or enzymes performing the primary cleavage of MSA, perhaps by blocking active sites involved with the sulphonate group of MSA. The result that MMSA was oxidised incompletely by MSA-grown cells suggested that such a reaction was occurring. For every mole of MMSA oxidised, 0.5 moles of oxygen were consumed.

The oxidation of MSA was performed only by cells grown on MSA (see section 7.4.2 and 8.3). Methanol-grown cells, however, were found to oxidise MMSA with the same stoichiometry as MSA-grown cells. This meant that the effect observed was probably unconnected with the MSA-specific enzymes. The MMSA used in the experiment was of relatively low purity (95%) and the oxidation observed may have been due to the 5% impurities present in the crystalline compound. Alternatively, the MMSA was oxidised by some other enzyme or enzymes common to MSA- and MeOH-grown cells.

7.6.4 Methane phosphonate (MPA)

The metabolism of the phosphorous analogue of MSA has been discussed (section 6.1) as a model for the

metabolism of MSA. Methane phosphonate was not oxidised by MSA-grown cells.

7.7 Incorporation of putative intermediates into MSA-grown cells

How a small quantity of a particular labelled compound is assimilated during growth on another unlabelled compound can produce information about the putative intermediates of the growth substrate. The contribution to cell carbon from various sources can be assessed using ^{14}C-labelled substrate supplements. For example, in the case of M2, this could have been valuable in determining whether methanol played a part in MSA metabolism.

The experiments were carried out with cells that were growing in chemostat culture, with 15 mM MSA as the main carbon and energy source. If methanol was an intermediate of MSA metabolism, it was expected that labelled carbon from small methanol supplements would either be incorporated as cell carbon, or be oxidised to CO_2, in the same proportions to a control experiment using a known intermediate. The situation if methanol was not an intermediate is more complex, but the end result would differ from that of the control.

Two putative intermediates were tried: methanol and formate. A control of formaldehyde was used, as the serine pathway was present, of which formaldehyde is the entry point. These substances were supplied, separately,

at 1 mM a chemostat culture of M2, limited by 15 mM MSA. The substrates were supplied at a dilution rate of 0.08 hr^{-1}. Labelled carbon was assayed for in the supernatant and pellet of culture samples spun down at 10,000 g, until the radioactivity in each fraction was constant.

The results showed a marked deviance from the those expected theoretically. Cell carbon from formaldehyde is higher than that of methanol and about the same as formate, with 54% being incorporated. This might seem to suggest that during MSA metabolism, little formaldehyde is oxidised and the majority is assimilated as cell carbon, and that methanol was not an intermediate, unlike formate. This would conform to the scheme proposed in section 7.3. However, it is just as conceivable that the control is not directly comparable. Formaldehyde and formate are used in other anabolic steps in bacteria apart from the serine pathway, including manufacture of purine bases (Brock *et al* 1984) where formaldehyde is used to add an aldehyde group to phosphoribosyl-glycinamide (Figure 7.7, page 185). Formate is also used in purine biosynthesis.

7.8 Discussion

The process by which MSA is metabolized in the facultative methylotroph M2 has not been fully characterised to date, but a summary of the information presented in this thesis is given below.

1) Growth on MSA produces a drop in pH from the formation of sulphuric acid, formed from sulphite cleaved from MSA. No energy is obtained from the oxidation of sulphite to sulphate. Growth proceeds under slightly reduced oxygen concentration, but no metabolism occurs under anaerobic conditions.

2) The oxidation of MSA proceeds to formate in MSA-grown cells and is not apparently initiated by MDH, or if MDH is involved, it is in conjunction with a modifier protein. Although formate dehydrogenase is present, further oxidation is slow, and this may be due to some difficulty involved in the transport of formate across the cell membrane.

3) The organism derives cell carbon from MSA via use of the serine pathway. It would seem likely that although RUBISCO can be detected, it plays no major part in the assimilation of carbon.

4) The enzyme or enzymes responsible for the primary degradation of MSA do not produce methane as a primary product, and M2 does not grow as a methanotroph. MSA-grown M2 will not perform epoxidation, indicating that a monooxygenase may not be present.

5) Either methanol or formaldehyde are the first intermediates in the pathway for the degradation of MSA. Studies with cyclopropanol and whole cells, or with purified enzyme(s) may provide more data.

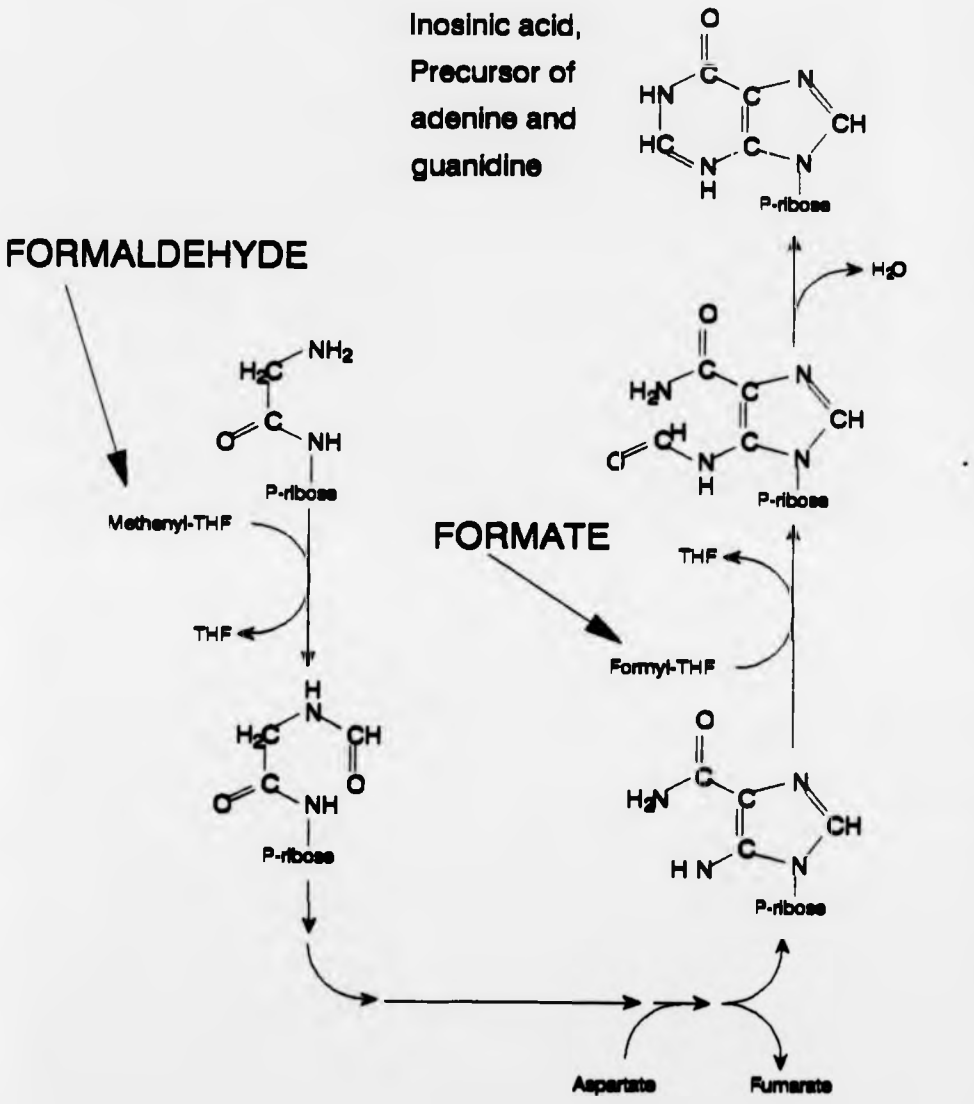


Figure 7.7. The biosynthesis of the purine ring. Methenyl-THF = N^5,N^{10}-methenyltetrahydrofolate, Formyl-THF = N^{10}-formyltetrahydrofolate, THF = tetrahydrofolate.

CHAPTER 8:
METABOLISM OF OTHER COMPOUNDS BY THE METHYLOTROPH M2

8.1 Introduction

Of the compounds that the strain M2 can use as sole sources of carbon and energy, methanol gave the highest growth rate under batch culture conditions (table 8.1.1).

Substrate	Doubling time hours
Methanol	4.93
Methane sulphonate	7.15
NH_2MSA	ND
Methylamine	6.22
Dimethylamine	6.32
Trimethylamine	5.04
Formate	15.8
Acetate	5.95

Table 8.1.1 Mean doubling times of strain M2 grown on C_1-compounds and acetate (All batch cultures grown with carbon source supplied at 10 mM). ND = not determined.

When considered under the classical definition of yield, M2 grew best on sodium glutamate, one mole theoretically providing 50.80 g dry weight of M2 (Table 8.1.2). However, a more descriptive measure of the organism's ability to use a compound for the production of biomass can be calculated, comparing dry weight to the number of carbon atoms available. Using this method, it was found that M2 grew most efficiently on methanol, followed by serine and acetate. Methane sulphonate (in a pH controlled medium) was as good a carbon source as glutamate, propionate and pyruvate. The yields show that although MinE and MinE-S are relatively low in nitrogen content compared to other media used for growth of methylotrophs (section 2.2.6), there seemed to be no advantage conferred when M2 grew on nitrogen-containing compounds, such as aminomethane sulphonate and methyl-

substituted amines.

The remainder of this chapter describes some of the aspects of the metabolism of a few of these compounds, predominantly methanol, formaldehyde, formate, methylamine and acetate, with regard to the information that can be gained from them in connection with the metabolism of MSA.

Substrate	Yield (g dry wt $mole^{-1}$)	Yield (g dry wt $[mole\ C]^{-1}$)
Methanol	16.10	16.10
Serine	43.90	14.63
Acetate	25.86	12.93
Methane Sulphonate	10.22	10.22
Glutamate	50.80	10.16
Propionate	30.40	10.13
Pyruvate	30.40	10.13
Formate	9.52	9.52
Aminomethane sulphonate	9.45	9.45
Lactate	19.14	6.38
Methylamine	6.30	6.30
Dimethylamine	11.40	5.70
Glucose	31.82	5.30
Trimethylamine	25.50	5.17
Thioacetamide	3.42	3.42
Trimethylsulphonium chloride	1.80	0.60
Formaldehyde	3.19	3.19
Succinate	6.22	1.56
Fructose	2.20	0.37
Sucrose	2.76	0.23
Citrate	1.40	0.20

Table 8.1.2 Apparent yields of biomass from the growth of M2 on C_n-compounds, expressed as g dry weight per mole substrate. Yield is also shown as g dry weight per mole carbon. All data taken from cultures supplied with 15 mM substrate. Yields calculated from 25 ml stationary phase culture samples, washed, and dried at 60 °C for two weeks or until weight was constant.

8.2 Metabolism of C_1 compounds

8.2.1 Sodium formate

The organism M2 will grow on formate as sole carbon and energy source with a doubling time of 15.8 hours and yielded 9.52 g dry weight $(mole)^{-1}$. The organism that appeared on MinE plus formate plates was definitely M2 and not contaminated with an autotrophic organism. Colonies from M2 were grown on MSA agar and subcultured into MinE containing formate. The formate-grown cells were serially diluted onto formate plates. Once single colonies had appeared, these were individually subcultured into MSA liquid medium. These subcultures grew at the same rate as M2, were morphologically similar and had the same growth range as M2 taken from stock cultures. Further subcultures, alternating between MSA and formate, retained the characteristics of M2.

It was thought that a separate method of assimilating carbon was in operation, probably a ribulose bis phosphate pathway. The RuBP pathway was identified as a putative candidate from the current literature on the metabolism of C_1 compounds, much of which ignores the biochemistry of formate assimilation in the absence of RUBISCO*. The likelihood of a formate reductase seemed remote from an energetic viewpoint, so at first it was thought that RUBISCO should have been present in formate grown cells (see section 7.2). The assay for RUBISCO was performed on whole cells and cell-free extracts, but did not consistently show a positive result

* The study of non-autotrophic formate assimilation in methylotrophs is discussed in the greatest detail in the work of Large and Quayle (1963), and this will be referred to again on p194

for the enzyme, much as MSA-grown cells had done (section 7.2.3). The amount of CO_2 fixed during growth on formate did not vary significantly from that fixed during growth on MSA or MMA, at about 20% of the total cell carbon.

The function of RUBISCO has been elucidated by more indirect methods. In an elegant experiment Kelly *et al.* (1979) prepared five flasks containing the autotrophic methylotroph *Thiobacillus versutus* (strain A2) grown on sodium [^{14}C]formate. The gaseous environment of the flasks was as follows:

1 Air - conventional flask stoppered with cotton wool
2 Air, sealed flask - Quickfit flask stoppered with a Subaseal
3 Air, sealed flask, CO_2 absorbed by KOH - the KOH is held separately from the medium in a well in the bottom of the flask
4 10% (v/v) CO_2 in air, sealed flask
5 5% (v/v) CO_2 in air, flushed through flask and medium

Flask 1 showed the growth rate and percentage of radio labelled cell carbon that would have been expected from a batch-grown methylotrophic autotroph. The organism derives some of its carbon from CO_2 in the atmosphere and more from the CO_2 resulting from the oxidation of formate. The organisms in flask 2 derive most of their cell carbon from formate, as the CO_2 in the atmosphere becomes depleted in the sealed flask. In flask 3 the CO_2 in the atmosphere is quickly depleted by potassium hydroxide, obliging the

thiobacilli to derive over 90% of their cell carbon from oxidised formate. Flask 4 has a local atmospheric concentration of CO_2 300 times higher than the Earth's. CO_2 from formate is diluted out, so less of this labelled carbon source is assimilated. Lastly, in flask 5, with an abundance of unlabelled CO_2 flushing through the medium, little of the cell carbon is derived from the labelled formate as most labelled CO_2 is flushed away with the excess gas. (table 8.2.1.1).

Flask number	Specific growth rate (h^{-1})	Formate assimilated [μmol (mg dry wt)$^{-1}$]	Percentage of cell-carbon from formate
1	0.096	24.2	65.9
2	0.084	30.4	83.0
3	0.093	33.2	90.7
4	0.143	8.1	22.0
5	0.146	2.8	7.7

Table 8.2.1.1 Short-term incorporation of ^{14}C-labelled carbon dioxide or formate by suspensions of Thiobacillus A2 previously grown on formate. Reproduced from Kelly et al (1979).

In a similar experiment using M2, specific growth rates and the percentage of cell-carbon from formate were identical to the control flask (flask 1), at 0.044 h^{-1} and 20% respectively. Three conclusions could be drawn from this:

1) That the binding of CO_2 to the RUBISCO of M2 is very strong, so absolute preference is given to internal CO_2 from formate, rather than external CO_2 (which in all cases has still to diffuse across a cell membrane).

2) That a phospho enol pyruvate carboxylase (or similar enzyme) immediately transforms all internal CO_2 to

oxaloacetate (or another non-volatile compound) as soon as it is formed.

3) That the fixation of CO_2 is not a means by which the strain M2 obtains cell carbon.

In order to resolve the question of the existence of RUBISCO in M2, proteins from a cell free extract of formate-grown M2 were separated on an SDS-PAGE gel and Western blotted with antibody raised in rabbits against the large sub-unit of wheat RUBISCO. It was found that the antibody bound to two proteins, of approximately the same weight as those binding in extracts of MSA-grown cells (section 7.2.3) The bound antibody did not react with the developer attached to a secondary antibody (section 2.10.7) with enough intensity to allow photography of this Western blot. The validity of these bands were, however, independently assessed. This dual binding of the antibody, coupled with previous blots in which other batches of the same antibody bound to proteins that were so large as to have not entered the gel, (probably not RUBISCO), calls into question the advisability of using antibody raised against wheat for detection of bacterial RUBISCO. Although the Western eventually revealed a protein of approximately the correct size, both in M2 and in a control of *Thiobacillus ferrooxidans*, the possibility of cross reaction with other non-carbon fixing proteins cannot be ruled out.

Using the premise that RUBISCO is not present, growth on CO_2 can be explained if the organism M2 is assumed to have a

system of formate metabolism similar to that of *Pseudomonas* AM1 (Large and Quayle, 1963). This organism achieves the fixation of formate via the use of a tetrahydrofolate formyl transferase (Figure 8.2.1.2).

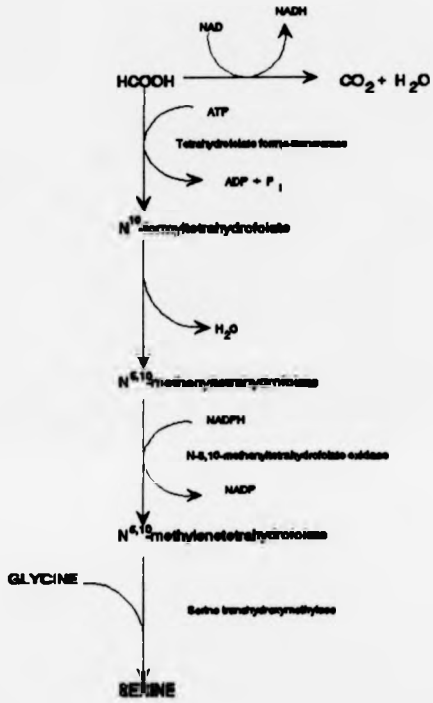


Figure 8.2.1.2 Reactions leading to the formation of serine from formate in the organism Pseudomonas AM1 (Large and Quayle, 1963).

Attached to tetrahydrofolate as N^{10}-formyltetrahydrofolate, the formate is reduced to $N^{5,10}$-methylenetetrahydrofolate, with the consumption of NADPH. Tetrahydrofolate is regenerated when the methylene group reacts with glycine to form serine. The serine pathway of carbon fixation then proceeds as normal. An organism with this pathway could grow

on formate without the use of RUBISCO, deriving energy from the oxidation of formate to CO_2 and water, and carbon from the fixation of formaldehyde, in the form of $N^{5,10}$-methylenetetrahydrofolate.

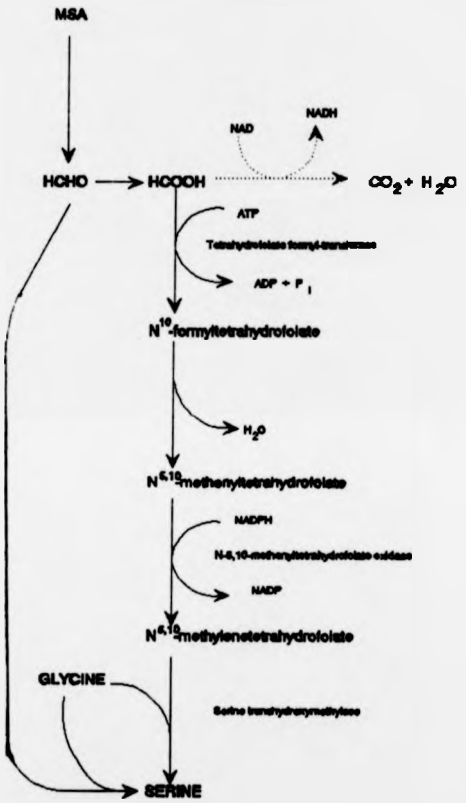


Figure 8.2.1.3 The metabolism of MSA by M2, involving the hypothetical action of tetrahydrofolate formylase forming a branched pathway, with no oxidation of formate.

The presence of enzymes in this pathway in M2 were not assayed for, but the hypothetical existence of this pathway does allow growth of M2 on formate, and could be used to rationalise the incomplete oxidation of MSA by MSA-grown

cells. If the enzymes in the pathway are induced during growth on MSA, and the binding of formate to tetrahydrofolate formylase is higher than that of formate dehydrogenase, an additional branch in the pathway would be formed, by-passing the oxidation of formate (Figure 8.2.1.3). Thus oxidation of MSA would be 0.5 moles of oxygen less than stoichiometry expected.

8.2.2 Monomethylamine

8.2.2.1 Growth and assimilation of carbon during growth on MMA

Monomethylamine was used as sole source of carbon and energy in maintainence media (section 2.6), and in the preparation of inocula. It had the advantage of not causing any great changes in the pH of the medium, allowing inoculation of MinE with a culture of approximately the same pH, and higher growth yield than MSA. Although methanol has a higher yield still and a quicker growth rate, crystalline MMA hydrochloride was easier to work with.

Cells growing on MMA had HPR activity comparable with that of MSA-grown cells, and obtained 21% of their cell carbon from CO_2 (figure 8.2.1.1). This indicated that MMA cells assimilated carbon via the serine pathway.

It should be noted that in most instances formate concentration is higher in the culture growing on 10 mM MMA compared to the 5 mM culture, indicating a substrate dependent effect, rather than one of experimental error. The

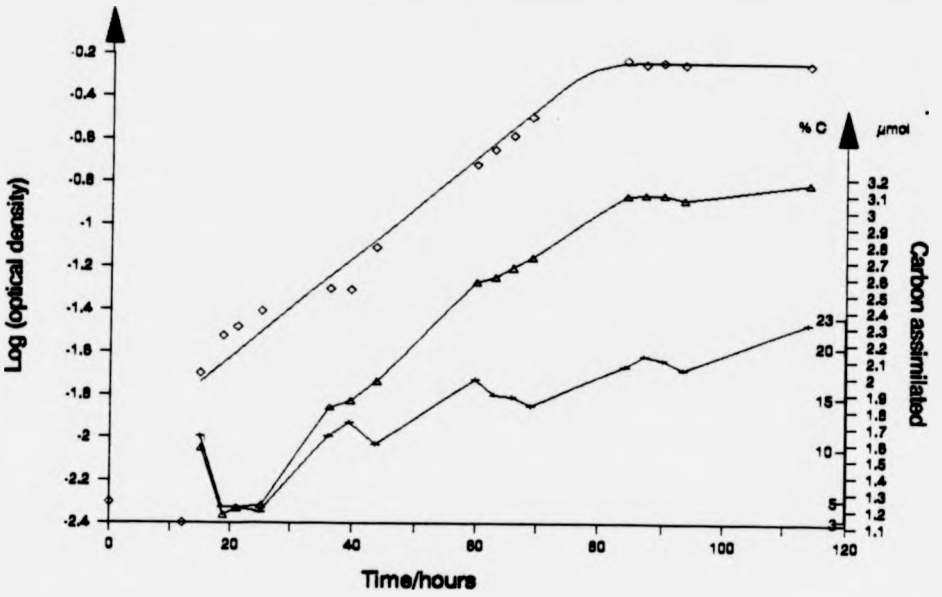


Figure 8.2.2.1 Growth and assimilation of carbon from CO_2 by M2 with 10 mM MMA as sole carbon and energy source. ◇ = OD_{440}, Δ = µmoles carbon assimilated, + = carbon assimilated expressed as a percentage of the all cell carbon.

figure clearly indicates that formate concentration falls during exponential growth. Four hourly measurements taken starting at 180 hours after inoculation established that no formate remained in the medium, showing that the organism does eventually metabolize the compound. The presence of exported formate suggests that this mode of growth is not restricted to metabolism of MSA (section 7.4.4).

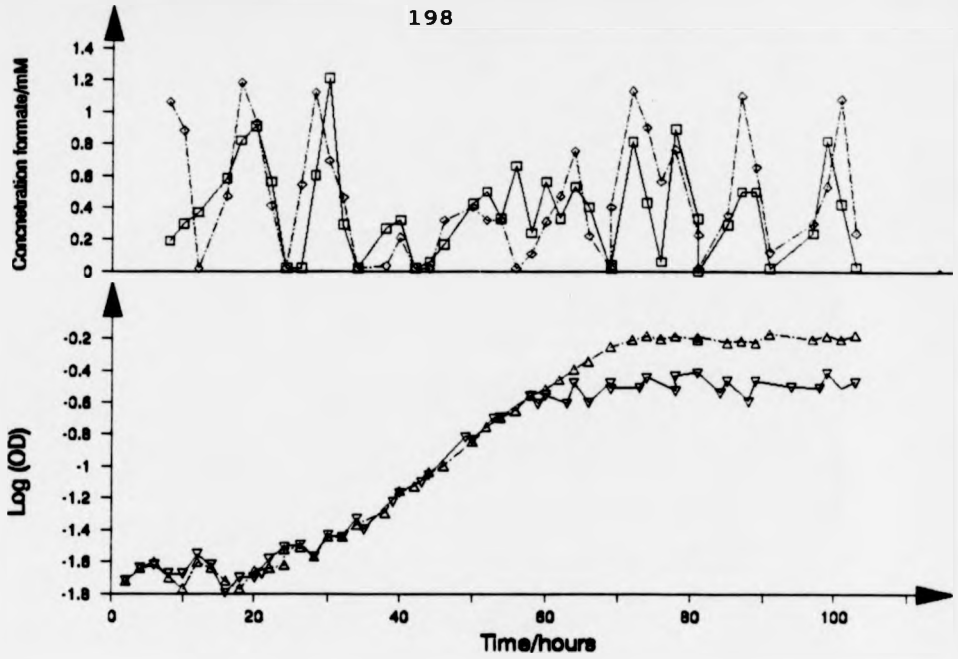


Figure 8.2.2.2 Concentration of formate in the media of cultures grown on 5 and 10 mM MMA. ▽ = Log(OD_{440}) 5 mM MMA culture, △ = Log(OD_{440}) 10 mM MMA culture, □ = formate concentration in 5 mM MMA culture, ◇ = formate concentration in 10 mM MMA culture.

8.2.3 Trimethylsulphonium chloride (TMS)

Pseudomonas strain MS was isolated by from soil by virtue of its ability to grow on TMS (Kung and Wagner, 1970). The metabolism of TMS was enabled by a tetrahydrofolate methyltransferase, catalysing the following reaction:

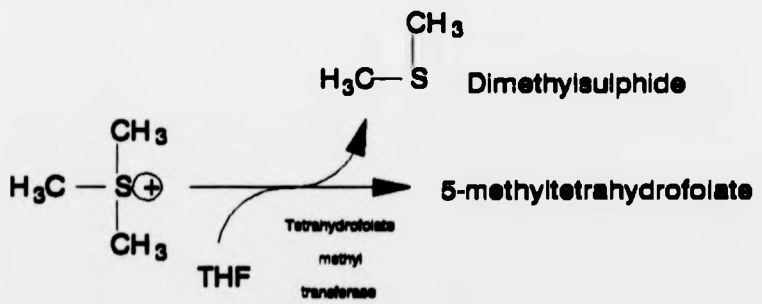


Figure 8.2.3 The metabolism of TMS by Pseudomonas species MS.

For every mole of TMS degraded by this enzyme, one mole of carbon is oxidised for energy or assimilated as cell carbon, and two moles are released into the medium as dimethyl sulphide.

The organism M2 was grown on 15 mM TMS in a Subasealed flask, but during the organism's growth no DMS could be detected in the headspace above the medium. This may suggest a different mode of metabolism than in *Pseudomonas* MS, yet the low yield of M2 on TMS (Table 8.1.2) suggests that only one of the methyl groups is assimilated, or that the culture was limited by some other factor.

8.2.4 Protein profiles of M2 grown on various substrates

Whole cells treated as in section 2.9.8.1 were run on SDS-PAGE gels. Although the procedure of creating protein profiles was tried at least twenty times, the bands the proteins of molecular weight of less than 40 kiloDaltons were always diffuse. This made associations with bands of a certain size with the substrates the cells were grown on difficult, though it was possible to postulate the involvement of proteins from the system involved in MMA metabolism with the metabolism of NH_2MSA (section 7.6.2) from such a gel.

The smearing of the bands was probably due to the presence of some storage compound in the cells. This manifested itself when the cells were lysed, by boiling or by the use of lysozyme, as a loose, white stringy mass distinct from the compact material of cell wall debris. It was not

possible to separate all of the white material from the solution of soluble proteins. Similar problems with storage material were noted in the preparation of DNA (section 6.3.6) and in the preparation of cells for oxygen electrode work (sections 7.4 and 8.3.2). The problem has since been solved by a different method of cell preparation (M. Davey and J.C. Murrell, University of Warwick, unpublished results).

8.2.5 Growth on sodium thiosulphate

No growth of M2 could be detected with thiosulphate as sole sulphur and energy source, nor could the oxidation of thiosulphate be detected during growth on MSA. This was assayed in the oxygen electrode and by the use of ^{14}C-labelled CO_2. When thiosulphate (10 mM) was added to batch cultures of M2 growing on MSA (10 mM), no additional biomass was noted compared to MSA alone, nor any differences in pH or carbon dioxide uptake.

The inability of M2 to use thiosulphate as an energy source implied that the organism probably does not gain any energetic advantage from the splitting of the carbon sulphur bond of MSA, as some *Thiobacillus* species do (e.g. *T. thioparus* strain E6 growing on DMS), nor from the subsequent oxidation of sulphite to sulphate. Oxidation studies showed that MSA-grown organisms did not biologically oxidise sulphite (section 7.4.5).

8.3 Oxidation studies

8.3.1 Introduction

Of the 21 compounds that serve as growth substrates for the organism M2, five C_1 compounds were selected for growth and oxygen electrode studies. Pyruvate was used as a comparative poly-carbon compound (Table 8.3.1).

Oxidation Substrate	Growth substrate					
	MSA	MeOH	HCHO	HCOOH	MMA	Pyr
MSA•	+	-	-	-	-	-
MeOH•	+	+	+	+	+	-
HCHO•	+	+	+	+	+	-
HCOOH•	*	+	*	+	*	-
NH_2MSA•		-	-	-	-	-
Formamide	-	-	-	-	-	-
MMSA	+	+	+	NT	+	-
ESA	-	-	NT	NT	-	NT
MPA	-	-	NT	NT	-	NT
MMS	-	-	NT	NT	-	NT
$MeNO_2$•	-	-	NT	NT	-	NT
MMA•	-	-	-	-	+	-
Pyruvate	-	-	-	-	-	+
Methane	-	-	NT	NT	-	NT
Ethanol	-	-	-	-	-	NT
Glucose•	-	-	-	-	-	+
Acetate•	-	-	-	-	-	NT
Ethylene	-	-	NT	NT	-	NT
Thiosulphate	-	-	-	-	-	-
Benzene sulphonate	-	-	NT	NT	-	-

Table 8.3.1 Compounds oxidised by M2 grown on MSA, methanol, formaldehyde, formate, pyruvate or MMA. NT = not tested. * = oxidation at medium concentration ≥16 mM. • = growth substrate.

8.3.2 Oxidation of substrates by methanol-grown cells

Cells taken directly from a methanol limited chemostat were unsuitable for use in the oxygen electrode as they displayed a high rate of endogenous respiration, concealing any substrate-dependent oxygen uptake. The level of endogenous

respiration was higher than that found in MSA-grown cells but was removed after washing three times in M2 buffer. If cells treated this way were used in electrode experiments, the addition of methanol produced a consumption of oxygen that exceeded the amount indicated by the stoichiometry of the complete oxidation of methanol. The "excess" oxidation did not alter the apparent stoichiometry of methanol oxidation by one or two moles, but by more than ten moles. The rate of this oxidation was equal to the endogenous respiration noted in unwashed cells.

This effect was negated by either shaking the washed cells at 30 °C for an hour, or a reiteration of washing (Table 8.3.2).

Cell treatment	Rate of endogenous oxidation nmol O_2 min^{-1} mg dry weight
None	21.88
Washed 3 times	21.53
Washed 6 times	0.10
Incubate 30 °C, 20 min	20.20
40 min	22.09
60 min	16.28
Incubate washed cells	0.15

Table 8.3.2 Rates of endogenous respiration of methanol-grown cells measured in the oxygen electrode after 7 different treatments.

The substrate oxidation range of the cells included methanol, formaldehyde and formate, but not MSA, MMA or any poly-carbon compound. This information indicates that the property of MSA metabolism was not a function of a broad substrate range for the MDH. It would appear that MSA metabolism is initiated by specialized enzyme(s), but a system in which the substrate range of M2 MDH is increased to include MSA by the action of a modifier protein can not

yet be discounted. Such a system has been shown to operate in *Methylophilus methylotrophus* and *Paracoccus denitrificans* by Long and Anthony (1991), where a periplasmic multimeric protein increased the affinity of MDH for higher alcohols, while lowering its affinity for formaldehyde.

Methanol-grown cells were capable of oxidising methanol at concentrations of up to 0.823 M, equivalent to adding 100 µl methanol straight from the stock bottle to the culture in the electrode, (table 8.3.3).

Concentration mM	Rate nmolO_2/min/mg dry weight
0.67	121.6
0.33	149.7
3.33	133.6
8.24	145.8
16.46	144.7
33.33	144.8
65.87	153.0
131.73	138.7
263.47	135.1
526.93	137.1
823.33	125.5

Table 8.3.3 Substrate inhibition of methanol oxidation by methanol-grown cells.

8.3.3 The oxidation of formate

Methanol- and formate-grown chemostat cultures of M2 were capable of stimulating oxygen consumption in the oxygen electrode in the presence of formate. The oxidation of formate by MeOH-grown cells had a Michaelis constant of 0.75 mM, while that of formate-grown cells was not determinable from the data available (table 8.3.4).

However, cells from chemostats limited by MMA or MSA were

not capable of stimulating formate oxidation unless the concentration of formate in the electrode exceeded 16 mM. This property has been noted in other methylotrophs, but a brief survey of the literature reveals that as many whole organisms can oxidise formate as those that appear not to do so. Whole cells of *Thiobacillus thioparus* E6 (Smith and Kelly, 1988a), *Pseudomonas* MS (Kung and Wagner, 1970) and many others oxidise formate with a very high apparent K_m, yet *Hyphomicrobium* X (Harder and Attwood, 1975) and *Xanthobacter flavus* H4-14 (W. Meijer, University of Groningen, personal communication) can effect the oxidation of formate, at concentrations in the nM to μM range.

The high concentration of formate needed to stimulate oxidation in M2 might reflect the repression of the formate dehydrogenase within the cell. However, FDH from MSA- or MMA-grown cells is easily detectable and has similar properties to that of other cells from other growth conditions (Table 8.4.2). It is more likely, therefore, that formate transport across the cell membrane(s) is repressed during growth on MSA or MMA. This hypothesis might also be used to explain the accumulation of formate in the media of batch cultures (figure 8.2.2.2). What purpose this repression might serve is not immediately apparent.

8.3.4 Oxidation of substrates by cells grown on other compounds

The data presented in table 8.3.4 show that the oxidation range of cells grown on C_1-compounds were essentially the

same: all could oxidise methanol, formate and formaldehyde with varying degrees of proficiency. The metabolic steps to the oxidation of MSA and MMA appeared to be induced only in cells exposed to these compounds as a growth substrate.

Cultures of M2 grown with pyruvate as sole source of carbon and energy did not have the ability to oxidise any of the C_1-compounds tested, but would oxidise glucose. This implies that the oxidation of C_1-compounds is a non-constitutive feature of the metabolism of M2.

Oxidation Substrate		Growth substrate					
		MSA	MeOH	HCHO	HCOOH	MMA	Pyr
MSA	K_m	0.02	-	-	-	-	-
	V_{max}	229	-	-	-	-	-
MeOH	K_m	0.11	0.10	0.13	0.14	0.14	-
	V_{max}	8020	9890	7650	7010	7900	-
HCHO	K_m	0.08	0.06	0.05	0.08	0.06	-
	V_{max}		150	302	216	138	-
HCOOH	K_m	*	0.75	*	nt	*	-
	V_{max}	*	1.38	*	nt	*	-
MMA	K_m	-	-	-	-	0.10	-
	V_{max}	-	-	-	-	109	-

Table 8.3.4 The Michaelis constants (in mM) and maximum rates of reaction (in nmolO_2 min^{-1} mg dry weight $^{-1}$) for cells grown on MSA, methanol, formaldehyde, formate, methylamine and pyruvate, and exposed to C_1 substrates in the oxygen electrode. * = oxygen consumption only at high (>16 mM) concentrations of substrate, - = no oxygen consumption.

8.4 Comparative enzyme assays

Chemostat cultures of M2 were grown at 80% of D_C, with MSA, methanol, formate, MMA or pyruvate as sole carbon source. Cell-free extracts of cells harvested from these chemostats were used in several enzyme assays outlined below. Endeavours to run a chemostat limited by formaldehyde did not succeed, and the small amounts of low activity extract obtained from batch cultures were used in qualitative rather than quantitative assays.

The assays used were for hydroxypyruvate reductase, hexulose phosphate synthase, formate dehydrogenase and, in some cases, RUBISCO. Methanol dehydrogenase was assayed at pH 10.0 with NAD as a cofactor, and at pH 9.0 with a dye-linked assay. As with MSA extracts (section 7.1.3.1), using the sodium tetraborate buffered MDH assay it was possible to obtain results for 1/v versus 1/S (Michaelis-Menton) and v versus v/S (Eadie Hoftsee) that deviated less from a best fit straight line than if a Tris buffer was used. The erratic nature of the RUBISCO assay when applied to M2 is discussed elsewhere in the text (sections 8.2.1 and 7.2.3).

The presence of the enzymes in the extracts are shown in table 8.4.1.

Methanol dehydrogenase is present in all extracts of cells grown on C1 compounds but not in pyruvate-grown cells. The metabolism of formate and MMA has been shown not to involve MDH in other bacteria, but in M2, MDH is induced. This provides supporting evidence for the supposition that the

Growth Substrate	Enzyme assay						
	HPR	HPS	MDH1	MDH2	MDH3	FDH	RUBISCO
MSA	+	-	-	NQ	+	+	-
MeOH	+	-	-	NQ	+	+	-
HCHO	+	-	-	NQ	+	+	NT
HCOOH	+	-	-	NQ	+	+	-
MMA	+	-	-	NQ	+	+	-
Pyruvate	+	-	-	-	-	-	-

Table 8.4.1 Presence of several enzymes found in cell free extracts of M2 grown on C1-compounds and pyruvate. NQ = positive under standard conditions, but non-quantifiable. NT = not tested. MDH1 was the assay for MDH at pH 10 with NAD+ as cofactor. MDH2 was the assay for MDH at pH 9.0 with TRIS buffer and PMS-linked activity. MDH3 was the assay for MDH at pH 9.0 with sodium tetraborate buffer and PMS-linked activity. For K_m and v_{max} of positive results, see table 8.4.2.

presence of MDH activity in MSA-grown cells is not a result of the metabolism of MSA. It would seem that the factors controlling the transcription and translation of MDH are more likely to be connected with compounds at the level of formaldehyde, formate or those of the carbon assimilation pathway than a more simple substrate-dependent control.

The key enzyme in the serine pathway, HPR, was induced during growth on all the substrates tested, including pyruvate. However, the Km of the enzyme in pyruvate-grown cells is far less than the constants in other types of cell (Table 8.4.2).

This may mean that the reduction of hydroxypyruvate has a role elsewhere in M2 cellular metabolism.

It has been suggested that the HPR of *Methylobacterium extorquens* AM1 has a second function in the oxidation of glycolate to glyoxylate - this reaction is necessary to enable use of the

Growth Substrate	Enzyme assay					
	HPR		MDH		FDH	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
MSA	4.40	1120	0.243	115	3.53	47
MeOH	4.04	1230	0.221	384	3.15	135
HCHO	3.57	978	0.189	104	ND	ND
HCOOH	4.14	898	0.183	97	0.54	53
MMA	3.82	1010	0.124	104	1.57	75
Pyruvate	0.86	31	0	0	0	0

Table 8.4.2 Michaelis constants (substrate concentration at half-maximal reaction rates, K_m) and maximum reaction rates for three enzymes found in soluble cell-free extracts of the organism M2. Units: HPR - K_m in mM, V_{max} in nmol NADPH/min/mg protein; MDH - K_m in mM, V_{max} in nmol DCPIP/min/mg protein; FDH - K_m in mM, V_{max} in nmol NAD/min/mg protein. ND = not determined, but present.

serine pathway for C_2 metabolism (Anthony, 1982; Dunstan *et al*, 1972). Only the reduction of glyoxylate to glycolate could be demonstrated in cell-free extracts, which has no apparent value *in vivo*. Further work by Chistoserdova and Lidstrom (1991) with the purified HPR of AM1 provided evidence that the enzyme is capable of both the oxidation of glycolate and the reduction of glyoxylate, dependent on *in vitro* conditions. This work provides some explanation of the activity of HPR in pyruvate-grown cells of M2. Only work at a molecular level (Eg detection of HPR mRNA) could show that both the activity associated with C_1 growth and that with acetate growth were due to the same enzyme: it is conceivable that the reductase activity noted in cell free extracts is attributable to another enzyme. This may account for the discrepancy in K_m values between the activity detected on C_1 compound-limited growth compared to pyruvate limited growth.

Formate dehydrogenase was found to be present in extracts

prepared from C1-grown cells. MSA-, methanol-, formaldehyde- and MMA-grown cells had similar FDH activity (Table 8.4.2) despite the low affinity for formate exhibited by these cells observed during oxidation experiments (table 8.3.1 and section 7.4.3)

8.5 Discussion

The enzymatic and oxidative properties of cells grown on C_1-compounds indicate that the metabolism of MSA is due to enzymes separate from those of methanol, formaldehyde, formate and methylamine metabolism. However, MSA-grown cells can perform transformations common to C_1-metabolism as a whole.

The presence of HPR and no other carbon assimilation enzymes in methanol, formaldehyde, MMA and formate grown cells suggests that the serine pathway is the main assimilation pathway for all C_1 compounds. The presence of HPR in pyruvate-grown cells indicates another role for the enzyme, although the lower values for K_m and V_{max} may indicate that a different enzyme was involved.

Formate appeared to have a different roles in the metabolism of all the C_1-compounds examined. FDH can be detected in all the C_1-compound-grown cells, but the ability to oxidise low concentrations of formate is restricted to cells grown on methanol and formate. An explanation must lie in the regulation formate transport, for some reason switched off during growth on MSA, MMA and HCHO. The possible energetic advantages of such a switch are hard to envisage.

The known metabolic routes of C_1 degradation are summarised in figure 8.5

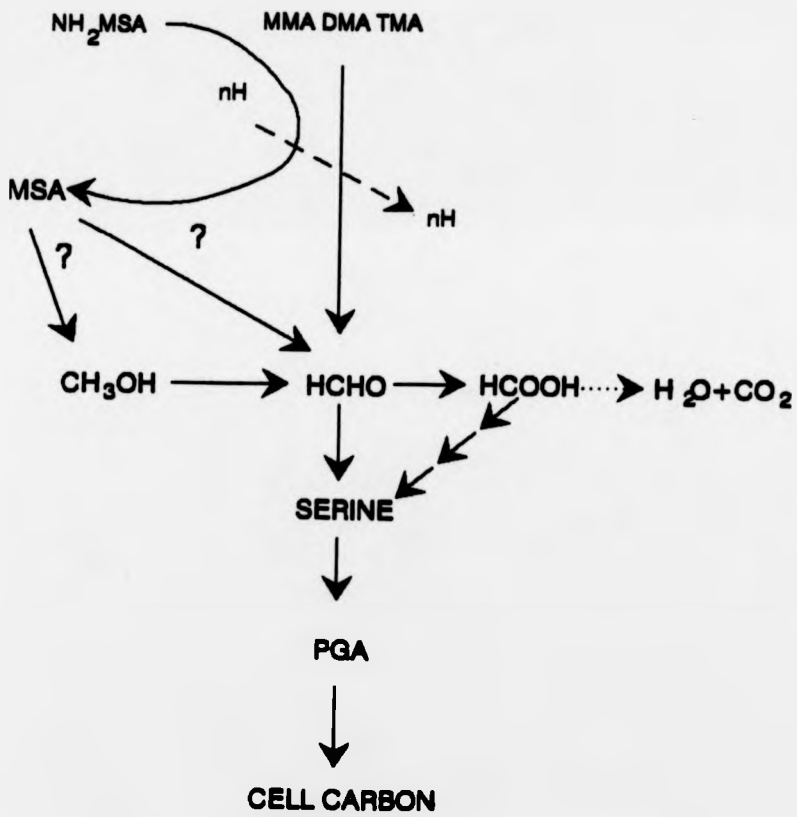


Figure 8.5 A summary of the known pathways of C_1-metabolism in the organism M2.

CHAPTER 9:
CONCLUDING REMARKS AND SUGGESTIONS FOR FURTHER STUDY

9.1 Carbon disulphide users

That the isolation procedure for CS_2 users failed was a reflection of the unusual culture techniques needed and the importance the work on MSA assumed. With time no doubt pure cultures would have been obtained, given the alacrity with which impure cultures used CDS. This work could have been of great interest to those involved with the treatment of chemical effluents. However, the tightening of laboratory regulations with regard to hazardous substances would probably have meant that characterisation of the organisms and their metabolic routes would be difficult.

9.2 The Harfoot culture collection

Although these isolates proved to be mixed cultures, the existence of microaerophillic thiobacilli capable of oxidation of DES might be of interest in the characterisation of soil or sediment sulphide transformations.

9.3 The strain M2 and MSA users

The current interest in the biogeochemistry of sulphur compounds has allowed the work on MSA users to continue. An interest has also been shown for the use of M2 in commercial waste treatment. Subsequent to this work, five positions at the University of Warwick have become involved with work on M2 or MSA microbiology. My own interest in the organism demands that every aspect of M2 biology needs further investigation, but the more important areas are outlined

below.

Strain M2 would appear to be a conventional facultative methylotroph with the unusual property of the metabolism of MSA. The K_m for HPR, MDH and FDH in cells grown on C_1-compounds appear consistent with those published concerning other methylotrophs using the serine pathway. The change in K_m for HPR when M2 was grown on pyruvate would seems to suggest another enzyme is induced during growth on this compound, of indeterminate function.

From the information so far available, the enzyme or enzymes that allow the metabolism of MSA seem to be induced solely by growth on methylated sulphonates (MSA and NH_2MSA), but further work will be needed to clarify this statement (Eg induction experiments with ESA, benzene sulphonates and other MSA analogues).

9.3.1 Growth on and oxidation of formate

Throughout the work, the use of formate in growth and oxidation experiments has resulted in observations that cannot be readily explained. In summary, these results were:

1) Formate is detected in the media of batch cultures with MSA or MMA as sole source of carbon and energy, but not in continuous cultures.
2) M2 will grow on formate, but has no RUBISCO.
3) MSA- and MMA-grown whole cells will not oxidise formate in the oxygen electrode, but possess FDH at levels equivalent to cells grown on other C_1 compounds. MeOH- and

formate-grown cells will oxidise formate.

4) Whole MSA-grown cells in the oxygen electrode will apparantly only oxidise MSA to formate, whereas the same cells will oxidise MeOH to CO_2 and H_2O.

Evidently the regulation of formate is influenced by the substrate on which the cells are grown, but at this point it is difficult to ascertain whether the regulation is at the level of formate import, FDH or by some other means entirely. A more focused study could provide more definite solutions to the problems encountered.

The presence of a tetrahydrofolate formyl transferase carrier system during growth on formate mentioned in chapter 8 is presently only a theorem concocted while writing this thesis. Assaying for the transferase enzymes involved in this system would be straightforward, using the methods described by Large and Quayle (1963).

9.3.2 Isolation and identification

A postulate mentioned in this thesis was that bacteria capable of growth on MSA are ubiquitous in the natural environment. The work needed to confirm this theorem has for the most part been completed. Second year undergraduates at the University of Warwick under the supervision of J.C. Murrell have isolated MSA users from a variety of British terrestrial and freshwater habitats. The isolation medium used was Min E, but the primary isolation substrate was sometimes methanol rather than MSA itself. It would thus seem probable that methylotrophs already held in culture

collections may have the ability to use MSA, and a screening programme may be of interest. The new bacterial isolates, though now stored as pure cultures, have yet to be fully characterised.

Marine microorganisms capable of growth on MSA have also recently been isolated in Dr Murrell's laboratory. These have a requirement for sodium chloride and can only be maintained in liquid culture. Thus it would appear that MSA users are indeed ubiquitous.

The strain M2 will soon be deposited in a culture collection, once an accurate determination of the G+C% for the organism has been completed. Numerical taxonomic tools such as 16S rRNA sequencing could be used to give a more empirical image of the organism's relationship to other methylotrophs, and with the eventual aim of the assignment of a genus and species name. Such work would involve the cooperation of a specialist laboratory and probably will not take place for some years.

9.3.3 Biochemistry

A post doctoral position at Warwick has been created to perform the task essential to the progression of MSA microbiology. This is the isolation and purification of the MSA "oxidase" protein or proteins. SDS-PAGE techniques different from those described in this thesis have shown that extra proteins are present in MSA-grown cells compared to methanol-grown cells. If the apparent lability of the MSA oxidising system (section 7.4) is circumvented, then many

possibilities will arise. These include the manufacture of MSA "oxidase"-specific DNA probes (as a result of N-terminal protein sequencing) for environmental evaluation of the presence of MSA users, probing in other type culture organisms etc. as well as more conventional protein biochemistry concerning the primary reactions involved in MSA metabolism.

As well as such an extensive project, many simpler aspects of M2 biochemistry need further examination. The effect of inhibitors on the oxidation of MSA by whole cells or cell-free extracts might yet provide information on the metabolism of MSA. Cyclopropanol has already been mentioned, but other structural analogues such as methyl methane thiosulphonate ($CH_3.S.SO_3.CH_3$) could also be useful.

The work on the putative RUBISCO activity of M2 could have reached a more satisfactory conclusion. A Western blot using monoclonal antibody raised against bacterial RUBISCO would provide a better indication of the expression of the protein in MSA- and formate-grown M2, as opposed to the polyclonal anti-wheat antibody method described in this thesis. The time needed to purify such a monoclonal antibody would necessitate the cooperation of another laboratory. However, work is proceeding at Warwick with a view to probing the DNA of M2 with a RUBISCO gene, providing some indication of the possible involvement of the enzyme in methylotrophic M2 metabolism. It may be that, like *Methylococcus capsulatus*, RUBISCO perform a function relate to energy balance rather than carbon assimilation.

9.3.4 Molecular biology

The molecular biology of M2 is in its infancy. V.L. McGowan (University of Warwick) has identified the MDH gene in M2, using a PCR method. MDH has also been identified in Western blots of MSA- and formate-grown M2, but not in cells form cultures limited by pyruvate. These results confirm biochemical assays, and would suggest that the MDH of M2 is not radically different from the MDH of other organisms. This easily identifiable MDH might serve as a good marker for gene regulation experiments, with a view to determining the switch between C_1 growth and growth on poly carbon compounds.

These results confirm biochemical assays, and would suggest that the MDH of M2 is not radically different from the MDH of other organisms. However, the DNA sequencing of the MDH gene cluster still has some value. I have gained a Royal Society European Exchange Fellowship to perform this task at the University of Groningen with Professor L. Dijkhuizen, with a view to producing mutants specifically deficient in the MDH enzyme. Production of MDH^- mutants by treatment with chemical mutagens might conceivably produce PQQ^- mutants, with unknown effects on the metabolism of MSA (Additionally, chemical mutagenesis performed by second- and third-year undergraduates has failed to produce satisfactory results). The characterisation of true MDH^- mutants would show clearly whether the "methanol" or "formaldehyde" pathway was in operation during growth on MSA.

The generation of vectors and other tools suitable for use

with M2 and similar organisms would allow the molecular characterisation of M2 to be initiated.

CHAPTER 10.
REFERENCES

ACKERMAN, D.G., HARO, M.T., RICHARD, G., TAKATA, A.M., WELLER, P.J., BEAN, D.J., CORNABY, B.W., MIHLAN, G.J. & ROGERS, S.E. (1980), Health impacts, emissions and emission factors for noncriteria pollutants subject to *de minimus* guidelines and emitted from stationary conventional combustion processes, U.S. Environmental Protection Agency, Report EPA-450/2-80

ANDREAE, M.O., BERRESHEIM, T.W., ANDREAE, T.W., KRITZ, M.A., BATES, T.S. & MERRILL, J.T. (1988), Vertical distribution of dimethylsulphide, sulphur dioxide aerosol ions and radon over the northeast Pacific Ocean, Journal of Atmospheric Chemistry, 6:149

ANEJA, V.P., OVERTON, J.H., CUPITT, L.T., DURHAM J.L. & WILSON, W.E. (1979a), Direct measurements of emission rates of some atmospheric biogenic sulfur compounds, Tellus, 31:174

ANEJA, V.P., OVERTON, J.H., CUPITT, L.T., DURHAM J.L. & WILSON, W.E. (1979b), Carbon disulphide and carbonyl sulphide from biogenic sources and their contributions to the global sulphur cycle, Nature (London), 282:493

ANEJA, V.P., OVERTON, J.H., CUPITT, L.T., DURHAM J.L. & WILSON, W.E. (1980), Measurements of emission rates of carbon disulphide from biogenic sources and its possible importance to the stratospheric aerosol layer, Chemical Engineering Communications, 4:721

ANON. (1986), Hazard data bank, sheet number 76 - carbon disulfide, Safety Practitioner, 4:16

ANTHONY, C. & ZATMAN, L.J. (1964), The microbial oxidation of methanol. 1. Isolation and properties of *Pseudomonas* M27, Biochemical Journal, 92:609

ANTHONY, C. (1975), The biochemistry of methylotrophic micro-organisms, Scientific Progress (Oxford), 62:167

ANTHONY, C. (1982), *The Biochemistry of Methylotrophs*, Academic Press, London, New York.

AYERS, G.P. & GRAS, J.L. (1991), Seasonal relationship between cloud condensation nuclei and aerosol methanesulphonate in marine air, Nature (London), 353:834

BAKER, S.C., KELLY, D.P. & MURRELL, J.C. (1991), Microbial degradation of methanesulphonic acid: a missing link in the biogeochemical sulphur cycle, Nature (London), 350:627

BAKKE, J.E., FIEL, V.J. & ZAYLSKIE, R.G. (1989), Isotope dilution assay for urinary methanesulfinic and methanesulfonic acids: Application to detection of methylthio turnover, Biomedical and Environmental Mass Spectrometry, 18, 1096

BAMFORTH, C.W. & QUAYLE, J.R. (1977), Hydroxypyruvate reductase activity in *Paracoccus denitrificans*, Journal of General Microbiology, 101:259

BANWART, W.L. & BREMNER, J.M (1976), Evolution of volatile sulphur compounds from soils treated with sulphur-containing organic materials, Soil Biology and Biochemical, 8:439

BARNARD, W.R., ANDREAE, M.O. & IVERSON, R.L. (1984), Dimethylsulfide and *Phaeocystis poucheti* in the south-eastern Bering Sea, Continental Shelf Results, 3:103

BARNARD, W.R., ANDREAE, M.O., WATKINS, W.E., BINGEMER, H. & GEORGII, H.W. (1982), The flux of dimethyl sulfide from the oceans to the atmosphere, Journal of Geophysical Results, 87:8787

BATES, T.S., CHARLSON, R.J. & GAMMON, R.H. (1987), Evidence for the climatic role of marine biogenic sulphur, Nature (London), 329:319

BECHARD, M.J. & RAYBURN, W.R. (1979), Volatile organic sulphides from freshwater algae, Journal of Phycology, 15:379

BIEDLINGMAIER, S. & SCHMIDT, A. (1986), Characterisation of the non-constitutive ethanesulfonate uptake in *Chlorella fusca*, Biochimica et Biophysica Acta, 861:95

BIEDLINGMAIER, S. & SCHMIDT, A. (1987), Uptake and utilization of sulfonic acids in the cyanobacterial strains *Anabaena variabilis* and *Plectonema* 73110, Z. Naturfforsch., C: Biosci., 42, (7-8):891

BIEDLINGMAIER, S., KOST, H.P. & SCHMIDT, A. (1986), Utilization of sulfonic acids as the only sulfur source for the growth of photosynthetic organisms, Planta, 169:518

BREMNER, J.M. & STEELE, C.G. (1978), Role of microorganisms in the atmospheric sulphur cycle, Advances in Microbial Ecology, 2:155

BROCK, T.D., SMITH, D.W. & MADIGAN, M.T. (1984), Appendix 3. Biochemical pathways, In: *Biology of Microorganisms*, Fourth Edition, Prentice-Hall Inc., Englewood Cliffs, New Jersey

BUTLER, R.G., ROTHSCHILD, B. & KELLER, J.R. (1969), Metabolism of carbon disulphide by *Thiobacillus thiooxidans*, Bacteriological Proceedings, p64, American Society for Microbiology.

CHALLENGER, F.(1951), Biological methylation, Advances in Enzymology, 12:429

CHARLSON, R.J., LOVELOCK, J.E., ANDREAE, M.O. & WARREN, S.G. (1987), Oceanic phytoplankton, atmospheric sulfur, cloud albedo and climate, Nature (London), 326:655

CHATFIELD, R.B., CHARLSON, R.J., FRIEND, J.P. & JAESCHKE, W. (1985), The transformation of sulfur and nitrogen in the remote atmosphere: Working group report, In: *The Biogeochemical Cycling of Sulfur and Nitrogen in the Remote Atmosphere*, Chapter 5, Galloway *et al.* (Eds.), Reidel Publishing Company

CHENGELIS, C.P. & NEAL, R.A. (1987), Oxidative metabolism of carbon disulfide by isolated rat hepatocytes and microsomes, Biochemica Pharmacology, 36:363

CHISTOSERDOVA, L.V. & LIDSTROM, M.E. (1991), Purification and characterization of hydroxypyruvate reductase from the facultative methylotroph *Methylobacterium extorquens* AM1, Journal of Bacteriology, 173:7228

CLINE, J.D. & BATES, T.S. (1983), Dimethylsulfide in the equatorial Pacific Ocean: A natural source of sulfur to the atmosphere, Geophysical Results Letters, 10:949

COCKS, A. & KALLEND, T. (1988), The chemistry of atmospheric pollution, Chemistry in Britain, 24:884 & 912

COLBY, J. & ZATMAN, L.J. (1972), Hexose phosphate synthase and tricarboxylic acid-cycle enzymes in bacterium 4B6, an obligate methylotroph, Biochemical Journal, 128:1373

COLBY, J., DALTON, H. & WHITTENBURY, R. (1979), Biological and biochemical aspects of microbial growth on C_1 compounds, Annual Review of Microbiology, 33:481

COLBY, J., STIRLING, D.I. & DALTON, H. (1977), The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath), Biochemical Journal, 165:395

COOK, A.M. & HUTTER, R. (1982), Ametryne and prometryne as sulphur sources for bacteria, Applied and Environmental Microbiology, 43 (4):781

COOK, A.M., DAUGHTON, C.G. & ALEXANDER, M. (1979), Benzene from bacterial cleavage of the carbon-phosphorous bond of phenylphosphonates, Biochemical Journal, 184:453

COURTAULDS plc (1987), Internal memorandum; substance analysis: Carbon disulfide, Personal communication, G. Marsh, (1987)

COURTAULDS plc (1984), *Carbon disulphide Code of Practice*, Seventh Edition, September 1984, Courtaulds plc, Coventry.

DACEY, J.W.H. & WAKEHAM, S.G. (1986), Oceanic dimethylsulfide: Production during zooplankton grazing on phytoplankton, Science, 233:1314

DAFT, J. (1987), Determining multifumigants in whole grains and legumes, milled and low-fat grain products, spices, citrus fruits and beverages, Journal of the Association of Off. Analytical Chemistry, 70:734

DALTON, H. & WHITTENBURY, R. (1976), The acetylene reduction technique as an assay for the nitrogenase activity in the methane oxidising bacterium *Methylococcus capsulatus* strain Bath, Archives of Microbiology, 109:147

DAUGHTEN, C.G., COOK, A.M. & ALEXANDER, M. (1979a), Biodegradation of phosphate toxicants yields methane or ethane on cleavage of the C-P bond, FEMS Microbiology Letters, 5:91

DAUGHTON, C.G., COOK, A.M. & ALEXANDER, M. (1979b), Bacterial conversion of alkylphosphonates to natural products via a carbon-phosphorous bond cleavage, Journal of Agricultural and Food Chemistry, 27:1375

de BONT, J.A.M., van DIJKEN, J.P. & HARDER, W. (1981), Dimethyl sulfoxide and dimethyl sulfide as a carbon and energy source for the growth of *Hyphomicrobium* S, Journal of General Microbiology, 127:315

DEPREZ, P.P, FRANZMANN, P.D. & BURTON, H.R. (1986), Determination of reduced sulphur gases in Antarctic lakes and seawater by gas chromatography after solid absorbent preconcentration, Journal of Chromatography, 362:9

DERIKX, P.J.L., OP DEN CAMP, H.J.M., VAN DER, DRIFT C., VAN GRIEVSEN, L.J.L.D. & VOGELS, G.D. (1990), Odorous compounds emitted during production of compost used as a substrate in mushroom cultivation, Applied and Environmental Microbiology, 56:176

DICKSON, D.M., WYN JONES, R.G. & DAVENPORT, J. (1980), Steady state osmotic adaptation in *Ulva lactuca*, Planta, 150:158

DIJKSTRA, M., FRANK, J., JONGEJAN, J.J.A. & DUINE, J.A. (1984), Inactivation of quinoprotein alcohol dehydrogenases with cyclopropane-derived suicide substrates, European Journal of Biochemistry, 140:369

DUBNOFF, J.W. & BORSOOK, H. (1948), Dimethylmethin and dimethyl-ß-propiothetin in methionine synthesis, Journal of Biological Chemistry, 176:789

DUNSTAN, P.M., ANTHONY, C. & DRABBLE, W.T. (1972), The role of glyoxylate, glycolate and acetate in the growth of *Pseudomonas* AM1 on ethanol and on C_1 compounds, Biochemistry Journal, 128:107

EAGLES, J. & KNOWLES, M.E. (1971), Trimethylsilyl esters of aromatic and aliphatic sulfonic acids, Analytical Chemistry, 43:1697

ECO, U. (1989), *Foucault's Pendulum*, Secker & Warburg, London.

FRANK, J. & DUINE, J.A. (1990), Methanol dehydrogenase from *Hyphomicrobium* X, Methods in Enzymology, **188**:202

GALLOWAY, J.N. (1985), Background paper: Sulphur and Nitrogen Deposition, In: *The Biogeochemical cycling of Sulfur and Nitrogen in the Remote Atmosphere*, Chapter 8, p145-175, Galloway et al. (Eds.), Riedel Publishing Company

GALLOWAY, J.N., CHARLSON, R.J., ANDREAE, M.O. & RODHE, H. (1985), Summary, In: *The Biogeochemical cycling of Sulfur and Nitrogen in the Remote Atmosphere*, Chapter 11, p215-233, Galloway et al. (Eds.), Riedel Publishing Company

GHISALBA, O., KUENZI, M., GERARDO, M.R.T. & SCHAR, H.-P. (1986), Biodegradation and utilisation of methylphosphates and methylphosphonates by specialized methylotrophs, In: *Abstracts of the 5th International Symposium on Microbial Growth on C1 Compounds*, August 11-16, 1986, Haren, The Netherlands, Duine J.A. and van Verseveld H.W. (Eds.), Free University Press, Amsterdam

GIBSON, J.A.E., GARRICK, R.C., BURTON, H.R. & McTAGGART, A.R. (1989), Dimethylsulfide concentrations in the ocean close to the Antarctic continent, Geomicrobiology Journal, **6**:179-184

GROENEVELD, A., DIJKSTRA, M. & DUINE, J. A. (1984), cyclopropanol in the exploration of bacterial alcohol oxidation, FEMS Microbiology Letters, **25**:311

GUENTHER, A., LAMB, B. & WESTBERG, H. (1989), U.S. National biogenic sulfur emissions inventory, In: *Biogenic Sulphur in the Environment*, pp. 14-30, Saltzman E.S. & Cooper W.J. (Eds.), ACS Symposium Series 393, American Chemical Society, Washington D.C.

HAINES, B., BLACK, M. & BAYER, C. (1989), Sulphur emissions from the roots of the rain-forest tree *Stryphnodendron exelsum*, In: *Biogenic Sulphur in the Environment*, Chapter 5, Saltzman E.S. & Cooper W.J. (Eds.), ACS Symposium Series 393, American Chemical Society, Washington D.C.

HARDER, W. & ATTWOOD, M.A. (1975), Oxidation of organic C_1 compounds by *Hyphomicrobium* spp., Antoinie van Leeuwenhoek, 41:421

HITCHCOCK, D.R. (1975), Dimethyl sulfide emissions to the global atmosphere, In: *Chemosphere*, No. 3, pp. 137-138, Pergammon Press, New York

HOEVEN, J.C.M. van der, MAK, J.K., FLOHR, P.M. & KNIPPENBERG, J.A.J. van (1986), *Review of Literature on Carbon Disulphide*, Bericht der NOTOX, 's-Hertogenbosch, und D.H.V., Raumplanung und Umwelt, Bereich Umweltschutz.

HUGH, R. & LEIFSON, E. (1953), O/F Test for acid from carbohydrates, Journal of Bacteriology, 66:24

IZUMI, Y., YOSHIDA, T. & YAMADA, H. (1990b), Purification and characterisation of serine-glyoxylate aminotransferase from a serine producing methylotroph, *Hyphomicrobium methylovorum* GM2, European Journal of Biochemistry, 190:285

JOLLIE, D. R. & LIPSCOMB, J. D. (1990), Formate dehydrogenase from *Methylosinus trichosporium* OB3b, Methods in Enzymology, **188:331**

JONES, T.D., WALSH, P.J., & ZEIGHAMI, E.A. (1985), Permissible concentrations of chemicals in air and water derived from RTECS entries: a "RASH" chemical scoring system, Toxicology and Industrial Health, **1:213**

KANAGAWA, T. & KELLY, D.P. (1986), Breakdown of dimethyl sulfide by mixed cultures and by *Thiobacillus thioparus*, FEMS Microbiology Letters, **34**, 13

KANAGAWA, T. & KELLY, D.P. (1987), Degradation of substituted thiophenes by bacteria isolated from activated sludge, Microbial Ecology, **13:47**

KANAGAWA, T. & MIKAMI, E. (1989), Removal of methanethiol, dimethyl sulfide, dimethyl disulfide and hydrogen sulfide from contaminated air by *Thiobacillus thioparus* TK-m, Applied and Environmental Microbiology, **55:555**

KANAGAWA, T., DAZAI, M. & FUKUOKA, S. (1982), Degradation of O,O-dimethyl Phosphodithioate by *Thiobacillus thioparus* TK-1 and *Pseudomonas* AK-2, Agricultural and Biological Chemistry, **46:2571**

KARAGOUNI, & SLATER, H. (1978), Growth of the blue-green alga *Anacystis nidulans* during washout from light- and carbon dioxide limited chemostats, FEMS Microbiology Letters, **4:295**

KATALYSE (1987), *BUND*, Oko-Institute, ULF: Chemieam Arbeitsplatz. Gefahrliche Arbeitsplatz. Gefahrliche Arbeitsstoffe, Berufskrankheiten und Auswege, Rowohlt, Reinbeck.

KELLY, D.P. (1988), Oxidation of sulphur compounds, In: *The Nitrogen and Sulphur Cycles*.
Society for General Microbiology Symposium 42:65

KELLY, D.P. & BAKER, S.C. (1990), The organosulphur cycle: aerobic and anaerobic processes leading to the turnover of C_1-sulphur compounds, FEMS Microbiology Reviews, 87:241

KELLY, D.P. & KEUNEN, J.G. (1984), In: *Aspects of Microbial Metabolism and Ecology*, Codd, C.A. (Ed.), p211-214, Academic Press, London.

KELLY, D.P. & SMITH, N.A. (1990), Organic sulphur compounds in the environment: Biogeochemistry, microbiology and ecological aspects, Advances in Microbial Ecology, 11:345

KELLY, D.P. & WOOD, A.P. (1982), Autotrophic growth of *Thiobacillus* A2 on methanol, FEMS Microbiology Letters, 15:229

KELLY, D.P. & WOOD, A.P. (1984), Potential for methylotrophic autotrophy in *Thiobacillus versutus* (*Thiobacillus* sp. strain A2), In: *Microbial Growth on C1 Compounds*, Proceedings of the 4th International Symposium, Crawford R.L. & Hanson R.S. (Eds.), American Society for Microbiology, Washington D.C.

KELLY, D.P., WOOD, A.P., GOTTSCHAL, J.C. & KUENEN, J.G. (1979), Autotrophic metabolism of formate by *Thiobacillus* strain A2, The Journal of General Microbiology, 114:1

KIENE, R.P. & CAPONE, D.G. (1988), Microbial transformations of methylated sulphur compounds in anoxic salt marsh sediments, FEMS Microbiology Ecology, 15:275

KIENE, R.P. & TAYLOR, B.F. (1988a), Biotransformations of organosulfur compounds in sediments via 3-mercaptopropionate, Nature (London), 332:148

KIENE, R.P. & TAYLOR, B.F. (1988b), Demethylation of dimethylsulfoniopropionate and production of thiols in anoxic marine sediments, Applied and Environmental Microbiology, 54:2426

KIENE, R.P. & VISSCHER, P.T. (1987), Production and fate of methylated sulfur compounds from methionine and dimethylsulfoniopropionate in anoxic salt marsh sediments, Applied and Environmental Microbiology, 53:2426

KIENE, R.P. (1988), Dimethyl sulfide metabolism in salt marsh sediments, FEMS Microbiology Ecology, 53:71

KIENE, R.P. (1990), Dimethyl sulfide production from dimethylsulfoniopropionate in coastal sea-water samples and bacterial cultures, Applied and Environmental Microbiology, 56:3292

KIENE, R.P., & BATES, T.S. (1990), Biological removal of dimethyl sulphide from sea water, Nature (London), 345:702

KING, G.F., RICHARDSON, D.J., JACKSON, J.B. & FERGUSON, S.J. (1987), Dimethylsulfoxide and trimethylamine-*N*-oxide as bacterial electron transport acceptors: use of nuclear magnetic resonance to assay and characterise the reductase system in *Rhodobacter capsulatus*, Archives of Microbiology, 149:47

KJAER, A. (1977), Low molecular weight sulphur-containing compounds in nature: A survey, Pure and Applied Chemistry, 49:137

KRAUSS, F. & SCHMIDT, A. (1987), Sulphur sources for the growth of *Chlorella fusca* and their influence on key enzymes of sulphur metabolism, Journal of General Microbiology, 133:1209

KUNG, H.-F. & WAGNER, C. (1970), Oxidation of C_1 compounds by *Pseudomonas* Sp. MS, Biochemical Journal, 116:257

LANG, E. & LANG, H. (1972), Spezifsche Farbreaktionzum direkten Nachweis der Ameisensäure, Zentralblat Analytika Chemie, 260:8

LARGE, P.J. & QUAYLE, J.R. (1963), Microbial Growth on C1-compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1, Biochemical Journal, 87:386

LEADBETTER, L., SIEBERT, K., SCHOBERT, P. & BOWIEN, B. (1982), Relationship between activities and protein levels of ribulosebisphosphate carboxylase and phosphoribulokinase in **Alcaligenes eutrophus**, FEMS Microbiology letters, 14:263

LONG, A.R. & ANTHONY, C. (1991), The periplasmic modifier protein for methanol dehydrogenase in the methylotrophs *Methylophilus methylotrophus* and *Paracoccus denitrificans*, Journal of General Microbiology, 137:2353

LOVELOCK, J.E. (1974), CS_2 and the natural sulphur cycle, Nature (London), 237:452

LOVELOCK, J.E., MAGGS, R.J. & RASMUSSEN, R.A. (1972), Atmospheric carbon disulphide and the natural sulphur cycle, Nature (London), 237:452

MAW, G.A. & du VIGNEAUD, V. (1948), Compounds related to dimethylthetin as sources of labile methyl groups, Journal of Biological Chemistry, 176:1037

NASH, T. (1953), The colorimetric estimation of formaldehyde by means of the Hantzsch reaction, Biochemical Journal, 55:416

NELSON, D.C & JANNASCH, H.W. (1983), Chemoautotrophic growth of a marine *Beggiatoa* in sulfide-gradient cultures, Archives of Microbiology, 136:262

LARGE, P.J. (1990), Microbial metabolism of C_1-nitrogen compounds other than cyanide, FEMS Microbiology Reviews, 87:235

LARGE, P.J., PEEL, D. & QUAYLE, J.R. (1961), Microbial growth on C_1-compounds. 2. Synthesis of cell constituents by methanol- and formate-grown *Pseudomonas* AM1, and methanol-grown *Hyphomicrobium vulgare*, Biochemical Journal, 81:470

LARGE, P.J., PEEL, D. & QUAYLE, J.R. (1962a), Microbial growth on C_1 compounds. 3: Distribution of radioactivity in metabolites of methanol-grown *Pseudomonas* AM1 after incubation with [^{14}C] methanol and [^{14}C] bicarbonate, Biochemical Journal, 82:243

LARGE, P.J., PEEL, D. & QUAYLE, J.R. (1962b), Carboxylation of phosphoenolpyruvate in methanol-grown *Pseudomonas* AM1, Biochemical Journal, 85:243

LAWTON, S.A. & ANTHONY, C. (1985), The role of cytochromes and blue copper proteins in the oxidation of methanol and methylamine in organism 4025, an obligate methylotroph, Journal of General Microbiology, 131:2165

LAY, M.D.S., SAUERHOFF, M.W. & SAUNDERS, D.R. (1986), Carbon disulfide, In: *Ullman's Encyclopedia of Industrial Chemistry*, 5th Edition, Vol. A 5, pp. 185-195, Verlag Chemie, Weiheim

NELSON, D.C. (1989), Physiology and biochemistry of filamentous sulfur bacteria, In: *Autotrophic Bacteria*, Schlegel H.G. & Bowien B. (Eds.), Chapter 5, Science Tech Publishers, Madison

NGUYEN, B.C., GAUDRY, A., BONSANG, B. & LAMBERT, G. (1978), Reevaluation of the role of dimethyl sulphide in the sulphur budget, Nature (London), 275:637

NRIAGU, J.O., HOLDWAY, D.A. & COKER, R.D. (1987), Biogenic sulfur and the acidity of rainfall in remote areas of Canada, Science, 237:1189

OWENS, J.D. & KEDDIE, R.M. (1969), The nitrogen nutrition of soil and herbage bacteria, Journal of Applied Bacteriology, 32:338

PEARCE, F. (1988), Plankton shares the blame for sulphur pollution, New Scientist, 11th February

PEEL, D. & QUAYLE, J.R. (1961), Microbial growth on C-1 compounds: isolation and characterisation of *Pseudomonas* AM1, Biochemical Journal, 81:465

PETERS, H.A., LEVINE, R.L., MATTHEWS, C.G., SAUTER, S. & CHAPMAN, L. (1986), Synergistic neurotoxicity of carbon tetrachloride/carbon disulphide (60/20 fumigants) and other pesticides in grain storage workers, Acta Pharmalogical et Toxicological Supplementum, 59:535

QUAYLE, J.R. (1961), Metabolism of C_1 compounds in autotrophic and heterotrophic microorganisms, Annual Review of Microbiology, 15:119

QUAYLE, J.R. (1972), The metabolism of one-carbon compounds by microorganisms, Advances in Microbial Physiology, 7:119

RAJAGOPAL, B.S. & DANIELS, L. (1986), Investigation of mercaptans, organic sulphides and sulphur inorganic compounds as sulphur sources for the growth of methanogenic bacteria, Current Microbiology, 14:137

RASMUSSEN, R.A. (1974), Emission of biogenic sulfide, Tellus, 26:254

RASMUSSEN, R.A., KHALIL, M.A.K., DALLUGE, R.W., PENKETT, S.A. & JONES, B. (1982), Carbonyl sulfide and carbon disulfide from the eruptions of Mount St Helens, Science, 215:665

ROBINSON, E. & ROBBINS, R.C., (1970), Gaseous sulfur pollutants from urban and natural sources, Journal of the Air Pollution Control Association, 20:23

RODHE, H. & ISAKSEN, I. (1980), Global distribution of sulfur compounds in the trophosphere estimated in a height/latitude transport model, Journal of Geophysical Results, 85:7401

SAIGNE, C. & LEGRAND, M. (1987), Measurements of methane sulphonic acid in Antarctic ice, Nature (London), 330:69

SAIGNE, C., KIRCHNER, S. & LEGRAND, M. (1987), Ion chromatographic measurements of ammonium, fluoride, acetate, formate and methanesulphonate ions at very low levels in Antarctic ice, Analytica Chimica Acta, 203:11

SHIMAMOTO, G. & BERK, R.S. (1979), Catabolism of taurine in *Pseudomonas aeruginosa*, Biochimica et Biophysica Acta, 569:287

SIEBURTH, J.M. (1961) , Antibiotic properties of acrylic acid, a factor in the gastrointestinal antibiosis of polar marine animals, Archives of Microbiology, 146:192

SIELER, H. (1983), Identification key for coryneform bacteria derived by numerical taxonomic studies, Journal of General Microbiology, 129:1433

SIELER, H., BRAATZ, R. & OHMAYER, G. (1980), Numerical cluster analysis of the coryneform bacteria from activated sludge, Zbl. Bakt. Hyg., I. Abt. Orig. C1:357

SIELER, H., OHMAYER, G. & BUSSE, M. (1977), Taxonomische untersuchungen an Gram-positiven coryneformen bakterien unter anwendung eines EDV programs zur berechnung von vernetzungdiagrammen, Zbl. Bakt. Hyg., I. Abt. Orig. A., 238:475

SIVELÄ, S. & SUNDMAN, V. (1975), Demonstration of *Thiobacillus*-type bacteria, which utilise methyl sulphide, Archives of Microbiology, **103:303**

SIVELÄ, S. (1980), *Dimethyl sulphide as a growth substrate for an obligately chemolithotrophic Thiobacillus*, In: *Commentationes Physico-Mathmaticai*, Dissert No. 1 (Simons, L., Ed.) pp. 1-69, Societas Scientareum Pennica, Helsinki

SMITH, A.L. & KELLY, D.P. (1979), Competition in the chemostat between an obligately and a facultatively chemolithotrophic thiobacillus, Journal of General Microbiology, **115:377**

SMITH, N.A. & KELLY, D.P. (1988a), Isolation and physiological characterisation of autotrophic sulphur bacteria oxidising dimethyl disulphide as sole source of energy, Journal of General Microbiology, **134:1407**

SMITH, N.A. & KELLY, D.P. (1988b), Oxidation of carbon disulphide as the sole energy source for the autotrophic growth of *Thiobacillus thioparus* strain TK-m, Journal of General Microbiology, **134:3041**

SMITH, N.A. & KELLY, D.P. (1988c), Mechanism of the oxidation of dimethyl disulphide by *Thiobacillus thioparus* Strain E6, Journal of General Microbiology, **134:3031**

SPARNINS, V.L., BARABY, G. & WATTENBERG, L.W. (1988), Effect of organosulfur compounds from garlic and onions on benzo(a)pyrene-induced neoplasia and glutathione S-transferase activity in the mouse, Carcinogenesis, 9:131

STEUDLER, P.A. & PETERSON, B.I. (1984), Contribution of gaseous sulphur from salt marshes to the global sulphur cycle, Nature (London), 311:455

STOIBER, R.E., LEGGETT, D.C, JENKINS, T.F., MURRMANN, R.P. & ROSE, W.I. (1971), Organic compounds in volcanic gas from Santiaguito volcano, Guatemala, Bulletin of the American Geological Society, 82:2299

SUYLEN, G.M.H. (1988), *Microbial Metabolism of Dimethyl Sulphide and Related Compounds*, Department of Microbiology and Enzymology of the University of Technology, Delft, The Netherlands

TAYLOR, B.F. & HOARE, D.S. (1969), A new facultative thiobacillus and a reevaluation of the heterotrophic potential of *Thiobacillus novellus*, Journal of Bacteriology, 100:487

TAYLOR, B.F. & KIENE, R.P. (1989), Microbial metabolism of dimethyl sulfide, In: *Biogenic Sulfur in the Environment* (Saltzman, E.S. and Cooper, W.J., Eds), pp. 202-221, ACS, Washington

TAYLOR, S. (1977), Evidence for the presence of ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase in *Methylococcus capsulatus* (Bath), FEMS Microbiology Letters, 2:305

THÖMEL, F. (1987), Synthesen mit Schwefelkohlenstoff, Chemiker-Zeitung, 111:285

THYSSE, G.J.E. & WANDERS, T.H. (1972), Degradation of n-alkane-1-sulphonates by *Pseudomonas*, Antoine van Leeuwenhoek, 38:53

THYSSE, G.J.E. & WANDERS, T.H. (1974), Initial steps in the degradation of n-alkane-1-sulphonates by *Pseudomonas*, Antoine van Leeuwenhoek, 40:25

TIMMERMAN, R.W. (1978), Carbon disulphide, In: *Encyclopedia of Chemical Technology*, Kirk, R.E. and Othmer, D.F. (Eds.), 3rd Edition, pp. 742-757, Wiley, New York, Chichester, Brisbane, Toronto.

TUOVINEN, H.O. & KELLY, D.P. (1973), Studies on the growth of *Thiobacillus ferrooxidans*. I. Use of membrane filters and ferrous iron agar to determine viable numbers, and comparison of $^{14}CO_2$-fixation and iron oxidation as measures of growth, Archiv für Mikrobiologie, 88:285

TURNER, S.M. & LISS, P.S. (1987), Dimethyl sulphide and dimethylsulphoniopropionate studies in European coastal waters, American Chemical Society, Division of Environmental Chemistry, 194th National Meeting (New Orleans), Vol. 27, No. 2, pp. 1-4

VAIRAVAMURTHY, A., ANDREAE M.O. & IVERSEN, R.L.(1985), Biosynthesis of dimethyl sulfide and dimethyl propiothetin by *Hymenomonas carterae* in relation to sulfur source and salinity variations, Limnology and Oceanography, 30:59

van DIJKEN, J.P. & HARDER, W. (1975), Growth yields of microorganisms on methanol and methane. A theoretical study, Biotechnology and Bioengineering, 17:15

van VERSEVELD, H.W. & STOUTHAMER, A.H (1978), Growth yields and efficiency of oxidative phosphorylation during autotrophic growth of *Paracoccus denitrificans* on methanol and formate, Archives of Microbiology, 118:21

VISSCHER, P.T. & van GEMERDEN, H. (1991), Photo-autotrophic growth of *Thiocapsa roseopersicina* on dimethyl sulfide, FEMS Microbiology Letters, 81:247

WAGNER, C., LUSTY, S.M., KUNG, H -F. & ROGERS, N.L. (1967), Preparation and properties of trimethylsulphonium-tetrahydrofolate methyltransferase, Journal of Biological Chemistry, 242:1287

WAGNER, C. & QUAYLE, J.R. (1972), Carbon assimilation pathways during growth of *Pseudomonas* AM1 on methylamine and *Pseudomonas* MS on methylamine and trimethylsulphonium salts, Journal of General Microbiology, 72:485

WAKEHAM, S.G., HOWES, B.L., DACEY, J.W.H., SCHWARZENBACH, R.P. & ZEYER, J. (1984), Biogeochemistry of dimethylsulphide in a seasonally stratified coastal pond, Geochimica Cosmochimica Acta, 51:1675

WESTBERG, H. & LAMB, B. (1984), In: *Environment Impact of Natural Emissions*, Aneja V.P. (Ed.), pp41-43, Air Pollution Control Association, Speciality Conference, Transactions, TR-2, Pittsburgh P.A.

WHITFIELD, F.B., SHEA, S.R., GILLEN, K.J. & SHAW, K.J. (1981), Volatile components from the roots of *Acacia pulchella* R. Br. and their effect on *Phytophthora cinnamomi* Rands, Australian Journal of Botany, 29:195

WICKBOLD, R. (1967), Über Zwischen-Producte beim biologischen Abbau eines geradkettigen Alkylbenzolsulfonats, In: *Chemistry, Physics and Application of Surface-active Substances*, C. Paquot (Ed), p903-912, Proceedings of the IVth International Congress on Surface-Active Substances, Brussels, 7-12 September 1964, Vol. III, Gordon and Breach Science Publishers, London, New York, Paris.

WOOD, A.P. & KELLY, D.P. (1977), Heterotrophic growth of Thiobacillus A2 on sugars and organic acids, Archives of Microbiology, 113:257

WOOD, A.P. & KELLY, D.P. (1989), Isolation and physiological characterisation of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and putative symbiont of *Thyasira flexulosa*, Archives of Microbiology, 152:160

ZATMAN, L.J. (1981), A search for patterns in methylotrophic pathways, In: *Microbial Growth on C1 Compounds*, Dalton H. (Ed.), pp. 42-54, Heydon & Son Ltd., London

ZEYER, J., EICHER, P., WAKEMAN, S.G. & SCHWARZENBACH, R.P. (1987), Oxidation of dimethyl sulfide to dimethylsulphoxide by phototrophic purple bacteria, Applied and Environmental Microbiology, 53:2026

ZINDER, S.H. & BROCK, T.D. (1978), Dimethyl sulfoxide as an electron acceptor for anaerobic growth, Archives of Microbiology, 116:35

ZINDER, S.H., DOEMAL, W.N. & BROCK, T.D. (1977), Production of volatile sulphur compounds during the decomposition of algal mats, Applied and Environmental Microbiology, 34:859

APPENDIX:
PUBLICATIONS

Posters:

BAKER, S.C. & KELLY, D.P. (1989), One-carbon compounds in the biogeochemical sulphur cycle, presented at the 6th International Symposium on Microbial Growth on C1-compounds, Göttingen, Germany, 20-25 August

Papers published in scientific periodicals (bound together at the end of this thesis):

KELLY, D.P. & BAKER, S.C. (1990), The organosulphur cycle: aerobic and anaerobic processes leading to the turnover of C1-sulphur compounds, FEMS Microbiology Reviews, 87:241

BAKER, S.C., KELLY, D.P. & MURRELL, J.C. (1991), Microbial degradation of methanesulphonic acid: a missing link in the biogeochemical sulphur cycle, Nature (London), 350:627

Papers in preparation:

BAKER, S.C., KELLY, D.P., & MURRELL, J.C. (1992), The oxidation of sodium methane sulphonate as the sole source of carbon and energy for the methylotrophic growth of strain M2, Journal of general Microbiology.